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Customer No.: **07278** Docket No: **21058/0206675-US0**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Mineo Yamakawa et al.

Serial No.: 10/750,141 Art Unit: 1754

Confirmation No.: 7926

Filed: December 31, 2003 Examiner: Daniel McCracken

For: METHODS OF PRODUCING CARBON NANOTUBES USING PEPTIDE OR NUCLEIC ACID MICROPATTERNING

- -

SECOND DECLARATION OF KAI WU, PH.D. PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Kai Wu, hereby declare as follows:

1. I am a citizen of the United States and over 21 years of age. I received a Bachelors of Arts degree in Chemistry in 1983 from New York University and a Ph.D. degree in Cell Biology and Genetics in 1990 from Cornell University. I am not an inventor on the present application but I am an employee of Intel Corporation, which is the assignee of the present application.

2. I have worked for over 18 years as a research scientist in the biotech and computer industry, have published 11 articles, have presented 3 posters, and have been an invited lecturer on 2 occasions. I am an inventor or co-inventor on 4 patents and 5 patent applications.

3. I have reviewed the file history of application serial number 10/750,141 (i.e., the present application or "the '141 application"), including the specification of the present application, the pending and amended claims (in the accompanying Amendment) in the present application, and the Office Action mailed January 8, 2008; and I make this declaration in support of the present application.

4. I understand that claims 1-6, 8-9, 11, and 15-21, and 39-41 of the '141 application are directed to a method for producing patterned arrays of carbon nanotubes on a substrate, wherein the distribution of the nanotubes is controlled by the prior attachment of catalyst nanoparticles to the substrate, and wherein the catalyst nanoparticles are directed to the substrate by way of their attachment to biomolecules which are aligned with the substrate to deposit the nanoparticles in a non-random fashion.

5. In understand that Examiner McCracken has requested a detailed description of how one of skill in the art could practice the claimed invention based on the content of the '141 application, including specific reference to well known procedures when necessary.

6. This declaration supplements my prior declaration submitted during prosecution of the '141 application on October 17, 2007 (hereinafter, the "First Wu Declaration").

7. It is my opinion that (1) the growth of carbon nanotubes on a substrate using catalyst nanoparticles, (2) molecular alignment with and/or attachment of biomolecules to a substrate, (3) attachment of e.g., proteins containing metal ions (e.g., ferritin) to biomolecules, and (4) conversion of these proteins to metal oxides with, for example, high temperature

calcination techniques are all a matter of routine biochemistry or materials science as disclosed in the '141 specification through citations to patents and literature references (*infra*). A detailed description of how one of skill in the art could practice the claimed invention based on the content of the '141 application and well known literature techniques is set forth in detail below.

Step 1: Attaching Catalyst Nanoparticles to Selected Locations on a Biomolecule with Defined Spacing Between the Nanoparticles

8. As stated in the my prior declaration, the specification of the '141 application discloses a number of methods for attaching catalyst nanoparticles to proteins, nucleic acids and other polymers so that there is a defined spacing between the nanoparticles (*See* First Wu Declaration, paragraph 8). One example described in the '141 specification states that "side-chain specific reagents can be used to create nanoparticle ... binding sites. For example, biotin-PE-maleimide ... can be reacted with cysteine residues of proteins ... or peptides ... or with sulfhydryl modified nucleotides. The biotin moiety ... can then be used to attach an avidin ferritin conjugated nanoparticle ... ." (*See* specification at para. [0026]).

9. The prior art offers a number of detailed methods for carrying out this step of the claimed processes. For example, Muir et al. *Chemistry & Biology* (1999) 6, R247-R256 (Exhibit A) describes how a series of polypeptides can be linked together such that cysteine residues are organized with a defined spacing. Using a technique known as native chemical ligation (*See* e.g., Muir, Figures 1(a) and 2(b)), a series of cysteine terminated oligopeptide thioesters (e.g., H_2N-Cys-$(Ala)_{15}$-SR) can be ligated to provide a polypeptide containing cysteine repeat units with a defined spacing (*See* Exhibit B; Figure 1, Step A). The polypeptide can then be reacted with an appropriate biotin derivative (e.g., Biotin-PE-maleimide) to selectively functionalize the cysteine residues with a biotin molecule (*See* Exhibit B; Figure 1, Step B). The functionalization

of cysteine residues with biotin was also well known in the art as disclosed in, for example, in Christopoulos et al. *Clin. Chem.* (1991) 37, 625-636 (Exhibit C; *See* e.g., Table 3 and page 626, col. 2, first full paragraph). The biotin functionalized polypeptide can then be reacted with ferritin-avidin to attach catalyst nanoparticle precursors to the biotin (*See* Exhibit B; Figure 1, Step C). Reaction of ferritin-avidin conjugates with biotin-derived proteins was also well known in the art at the time the '141 application was filed (*See* e.g., Exhibit C, page 631, col. 2, fourth paragraph, and Table 2, entry 3).

10. According to the three step process outlined above and illustrated in Figure 1, one of skill in the art could use the '141 disclosure and techniques that were well known in the art (e.g., Muir and Christopoulos) to attach catalyst nanoparticle precursors (e.g., ferritin) to selected locations on a biomolecule (e.g., cysteine residues of a polypeptide) with defined spacing between the nanoparticles. I declare that the synthesis of the nanoparticle-derived biomolecule could be accomplished with no more than routine experimentation.

Step 2: Attaching the Biomolecules to the Substrate

11. As stated in the my prior declaration, the specification of the '141 application discloses a number of methods for attaching a biomolecule to a substrate (*See* First Wu Declaration, paragraph 12). One example described in the '141 specification states that "the substrate can be patterned with a thin film of gold, using photo- or electron beam lithography, show masking or microcontact printing ... . Thiol modified nucleic acids ... can be covalently bonded to the gold patches on the substrate." (*See* specification at para. [0040])

12. The prior art offers a number of detailed methods for carrying out this step of the claimed processes. For example, Wittstock et al. *Langmuir* (2002) 18, 9485-9493 (Exhibit D) describes how the N-terminus of a polypeptide can be functionalized with sulfur moiety and

subsequently bound to a gold surface (*See* Exhibit D, page 9490, col. 2, 2nd full paragraph and page 9491, Schemes 1 and 2). This process is outlined in Figure 2 (Exhibit E), wherein the polyalanine biotin-avidin-ferritin conjugate from Figure 1, step C is reacted with SATA to form an N-terminal thioester. The thioester can then be deacylated with hydroxylamine to provide a thiol group at the N-terminus. This thiol group can them be chemiabsorbed on a gold surface. In the presently claimed method, the gold surface can be micropatterned onto, for example, a silicon substrate using lithography techniques. Accordingly, the polyalanine biotin-avidin-ferritin conjugate would be attached to selected areas on the substrate.

13. According to the process outlined above and illustrated in Figure 2, one of skill in the art could use the '141 disclosure and techniques that were well known in the art (e.g., Wittstock) to attach the biomolecule (e.g., ferritin derived polypeptide) to a substrate (e.g., a substrate patterned with gold). I declare that the attachment of the biomolecule to the substrate could be accomplished with no more than routine experimentation.

Step 3: Aligning the Biomolecule with a Substrate so that the Catalyst Nanoparticles are Aligned with the Substrate in a Non-Random Fashion

14. As stated in the my prior declaration, the specification of the '141 application discloses a number of methods for aligning a biomolecule with a substrate to further align the catalyst nanoparticles with the substrate in a non-random fashion (*See* First Wu Declaration, paragraph 10). For Example, "proteins can be aligned using any known molecular alignment method, such as molecular combing, optical tweezers, microfluidic flow magnetic fields, free flow electrophoresis, etc." (*See* specification at para [0075], lines 1-3).

15. The prior art offers a number of detailed methods for carrying out this step of the claimed processes. For example, Bensimon et al. U.S. Patent No. 6,548,255 (Exhibit F)

describes how macromolecules, including proteins (*See* Exhibit E, col. 7, line 64 to col. 8, line 6), can be aligned on the surface of a support using molecular combing. According to the methods of Bensimon, a protein is anchored onto a surface S (e.g., a gold surface; *See* Exhibit F, col. 7, lines 33-41), and the free part of the molecule is stretched by a meniscus, preferably an air water meniscus (*See* Exhibit F, col. 2, line 38-45). Because the meniscus moves in a uniform direction via, for example, evaporation of water, the bound proteins become aligned along the direction of evaporation. The Benmison process can be applied to the polyalanine biotin-avidin-ferritin conjugate bound to a micropatterned gold surface as shown in Figure 2. Accordingly, the polyalanine biotin-avidin-ferritin conjugates would be aligned with the substrate to that the ferritin nanoparticles would be non-randomly aligned with the substrate.

16. According to the process outlined above and illustrated in detail in Benmison, one of skill in the art could use the '141 disclosure and techniques that were well known in the art to align the biomolecule (e.g., ferritin derived polypeptide) with a substrate (e.g., a substrate patterned with gold) using molecular combing. Such biomolecule alignment would inherently align the catalyst nanoparticles with the substrate in a non-random fashion. I declare that the alignment of the biomolecule with the substrate could be accomplished with no more than routine experimentation.

Step 4: Removing the Biomolecules such that the Nanoparticles Attach to the Substrate at a Biomolecule Directed Site

17. As stated in the my prior declaration, the specification of the '141 application discloses a number of methods for removing the biomolecules such that the nanoparticles attach to the substrate at a biomolecule directed site (*See* First Wu Declaration, paragraph 14). For Example, "calcinations [of ferritin] in air at 800 C for 5 min, removes the ferritin shell and

oxidizes the iron core, resulting in discrete production of Fe_2O_3 nanoparticles ... of about 1.5 nm average size that are suitable for catalytic growth of SWNTs" (*See* specification at para [0028], lines 5-10).

18. The prior art offers a number of detailed methods for carrying out this step of the claimed processes. For example, Dai et al. *Acc. Chem. Res.* 2002, 35, 1035-1044 (Exhibit F) describes how stable solutions of artificial ferritin can be deposited onto flat substrates, and how subsequent calcinations in air leads to Fe_2O_3 nanoparticles bound to the surface of the substrate (*See* Exhibit F, page 1037, first full paragraph). The Dai method can be applied to the presently claimed invention using the polyalanine biotin-avidin-ferritin conjugates previously aligned using the molecular combing techniques of Benmison. Calcination of the aligned polyalanine biotin-avidin-ferritin conjugates would remove the biomolecule and lead to Fe_2O_3 nanoparticles deposited on the substrate at the location of each aligned and protein bound ferritin molecule. Thus, the Fe_2O_3 nanoparticles would be attached to the substrate at a site directed by the alignment of the ferritin nanoparticle precursors.

19. According to the process outlined above and illustrated in detail in Dai and Benmison, one of skill in the art could use the '141 disclosure and techniques that were well known in the art to remove the biomolecules (e.g., polyalanine biotin-avidin-ferritin conjugates) such that the nanoparticles (e.g., Fe_2O_3 nanoparticles) attach to the substrate at a biomolecule directed site. I declare that this attachment of catalyst nanoparticles to a substrate at a biomolecule directed site could be accomplished with no more than routine experimentation.

Step 5: Producing Substrate Attached Carbon Nanotubes on the Catalyst Nanoparticles With Non-Random Distribution of the Nanotubes.

20. As stated in the my prior declaration, the specification of the '141 application discloses a number of techniques for producing substrate attached carbon nanotubes from catalyst nanoparticles that are bound to a substrate. (*See* First Wu Declaration, paragraph 16). For Example, "typically, catalyst nanoparticles are used in combination with chemical vapor deposition (CVD) techniques, by flowing a hydrocarbon gas (e.g., CH4, C2H4) through a catalyst containing tube reactor at temperatures of about 500 to 1000 C, using H2 gas co-flow to provide reducing conditions." (*See* specification at para [0022], lines 4-11).

21. The prior art offers a number of detailed methods for carrying out this step of the claimed processes. For example, Dai et al. *Acc. Chem. Res.* 2002, 35, 1035-1044 (Exhibit F) describes how carbon nanotubes can be synthesized by CVD by heating a catalyst material (Fe_2O_3 nanoparticles) in a furnace and flowing a hydrocarbon gas through a tube reactor for a period of time. (*See* Exhibit F, page 1036, col.1 – col. 2, bridging paragraph). Using the CVD methods disclosed in Dai, patterned growth of nanotubes can be achieved by positioning catalyst materials in arrayed fashions on high surface area materials and applying the aforementioned CVD techniques. Accordingly, Fe_2O_3 nanoparticles deposited on a substrate by calcination of aligned polyalanine biotin-avidin-ferritin conjugates could serve as catalysts for carbon nanotube growth using the CVD techniques of Dai.

22. According to the process outlined above and illustrated in detail in Dai, one of skill in the art could use the '141 disclosure and techniques that were well known in the art to produce substrate attached carbon nanotubes on the catalyst nanoparticles with non-random distribution of the nanotubes. I declare that this non-random growth of carbon nanotubes could be accomplished with no more than routine experimentation.

23. Based on the foregoing remarks, I declare that the '141 specification, in combination with techniques that were well known in the art prior to the filing of the '141 application, enables one of skill in the art how to make and use the invention embraced by the current claims without undue experimentation.

24. I further declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the instant application or any patent issued thereupon.

10/01/2008
Dated

Kai Wu, Ph.D.

EXHIBIT A

Peptide ligation and its application to protein engineering

Graham J Cotton and Tom W Muir

The ability to assemble a target protein from a series of peptide fragments, either synthetic or biosynthetic in origin, enables the covalent structure of a protein to be modified in an unprecedented fashion. The present technologies available for performing such peptide ligations are discussed, with an emphasis on how these methodologies have been utilized in protein engineering to investigate biological processes.

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http://biomednet.com/elecref/1074552100 6R0247

1074-5521/99/$ – see front matter

Introduction

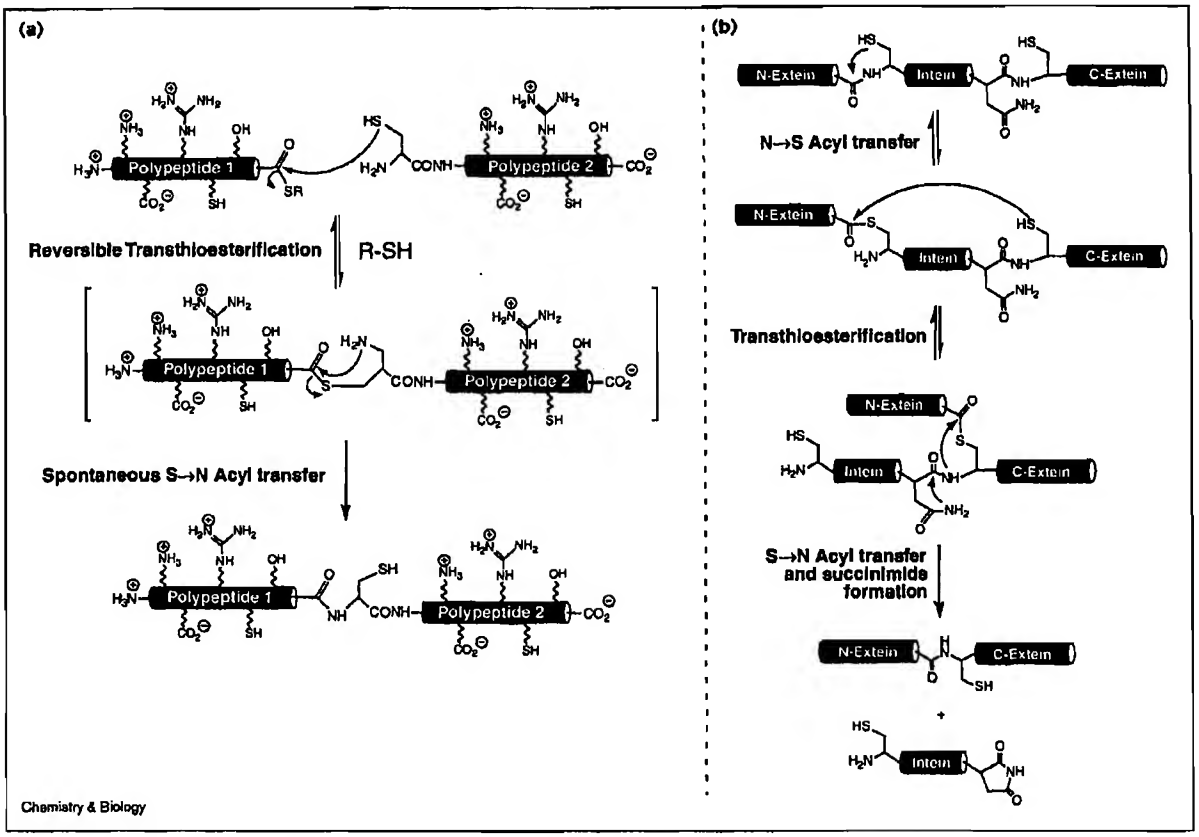
With the genetic blueprint of several organisms already available and the completion of the human genome project apparently well within sight, we are now facing a challenge worthy of a new millennium: characterization of many tens of thousands of novel gene products at the chemical and biological levels. Often referred to as the 'proteome project', this is an enormously daunting undertaking, particularly given the emerging picture of complexity in biological processes. It seems likely that both established and novel technologies will have to be brought to bear on the problem, the latter probably requiring the input of researchers from many branches of science and engineering. Chemistry, and in particular organic chemistry, is well poised to make a huge impact in this area, both through the use of small-molecule probes of biological processes [1] and, as will be the focus of this review, through the direct chemical manipulation of protein molecules. Precisely altering the covalent structure of a protein using synthetic chemistry provides a freedom in protein engineering unattainable by standard site-directed mutagenesis techniques. Accordingly, the development of approaches for the site-specific incorporation of unnatural amino acids into proteins has received considerable attention in recent years, and several biosynthetic and synthetic strategies are now available for this purpose [2–6].

This review will focus on just one of these technologies, namely the 'peptide ligation' approach [4,6]. Peptide ligation actually refers to a suite of approaches, all of which allow fully unprotected polypeptide building blocks to be regioselectively joined together in aqueous solution to create a target protein molecule. Ligation can be achieved either chemically [4], by incorporating unique functionalities at the amino and carboxyl termini of the peptide segments, or enzymatically using sophisticated reverse-proteolysis techniques [3,5]. The attraction of these modular ligation approaches is that they allow a selected region of a long protein sequence to be chemically manipulated in a manner analogous to a synthetic peptide, enabling proteins to be site-specifically modified in an hitherto unprecedented fashion. In this article, we will briefly review some recent technological developments in the peptide-ligation field and then attempt to illustrate the extraordinary potential of this area in protein engineering through a series of examples.

Bridging peptide chemistry and protein biotechnology

There are several different chemical and enzymatic ligation approaches available for the assembly of proteins

Figure 1



Comparison of the mechanisms of **(a)** native chemical ligation and **(b)** protein splicing.

from unprotected peptides (these have been reviewed extensively [3,4,6]). Indeed, small proteins and protein domains are now readily accessible to total synthesis, which is emerging as the method of choice for the rapid generation of these molecules [6]. Difficulties associated with the solid-phase peptide synthesis (SPPS) of peptides longer than ~50 amino acids do, however, render the assembly of large protein targets (> 100 residues) a significant challenge using only two synthetic peptide segments. One solution to this problem has been to link together three or more synthetic peptides either using a combination of orthogonal ligation approaches [7–9] or through the sequential use of the same methodology [9–12]. Only a handful of proteins, however, have been prepared using these strategies to date, primarily because they are technically demanding to perform. Perhaps as a consequence of this, recent developments in the field have centered on an alternative approach in which a combination of synthetic peptides and recombinantly derived polypeptides are used as the ligation building blocks. In principle, this semisynthetic strategy should allow synthetic access to extremely large protein systems by ligating short synthetic peptides (containing the chemical probe of interest) to much larger recombinant polypeptides. Pioneering work in this area includes the use of conformationally assisted condensation reactions [3], oxime and hydrazone bond forming ligation chemistries [13–15] and engineered peptide ligases [5].

The well-established 'native chemical ligation' method [16] has proved pivotal for many of the recent developments in protein semisynthesis. As illustrated in Figure 1a, the first step in native chemical ligation involves the chemoselective reaction that occurs at physiological pH between a peptide fragment containing an amino-terminal cysteine residue and a second peptide fragment containing an $^{\alpha}$thioester group. This initial transthioesterification reaction is then followed by a rapid intramolecular S→N acyl shift to generate an amide bond at the ligation junction. Note, additional cysteine residues

are permitted in one or both peptide segments because of the reversible nature of the initial transthioesterification step. This reaction has been widely used for ligating two synthetic peptide fragments together [6], where the necessary reactive functionalities can be incorporated into the fragments during SPPS. Significantly, the necessary reactive groups can now be incorporated in a general fashion into recombinant proteins by utilizing new directives in protein engineering.

Techniques for producing recombinant amino-terminal cysteine proteins, for use in native chemical ligations, have been established for some time [17]. It is only recently, however, that the requisite technologies have been developed that enable recombinant $^{\alpha}$thioester proteins to be generated. This breakthrough was achieved by manipulating a naturally occurring biological phenomenon known as protein splicing [18], a process that bears remarkable similarities to native chemical ligation (Figure 1). Protein splicing is a post-translational process in which a precursor protein undergoes a series of intramolecular rearrangements that result in precise removal of an internal region, referred to as an intein, and ligation of the two flanking sequences, termed exteins (Figure 1b). Although there are, with one exception (see below), no sequence requirements in either of the exteins, inteins are characterized by several conserved sequence motifs and approximately one hundred members of this protein domain family have now been identified (for a comprehensive listing, see www.neb.com/neb/Frame_tech.html).

The first step in protein splicing involves an N→S (or N→O) acyl shift in which the N-extein unit is transferred to the sidechain SH or OH group of a conserved Cys/Ser/Thr residue, always located at the immediate amino terminus of the intein. Note, it has been speculated that the intein structure provides the driving force for this thermodynamically unfavorable rearrangement by twisting the scissile amide-bond into a higher energy conformation [19]. The entire N-extein unit is then transferred to a second conserved Cys/Ser/Thr residue at the intein-C-extein boundary (+ 1 position) in a transesterification step. The resulting branched intermediate is then resolved through a cyclization reaction involving a conserved asparagine residue at the carboxyl terminus of the intein. The intein is therefore excised as a carboxy-terminal succinimide derivative. In the final step, an amide bond is formed between the two exteins following an S→N (or O→N) acyl shift, a step reminiscent of native chemical ligation. These mechanistic insights have led to the design of a number of mutant inteins that can only promote the first step of protein splicing [20–23]. Proteins expressed as in-frame amino-terminal fusions to one of these engineered inteins can be cleaved by thiols via an intermolecular transthioesterification reaction to generate the recombinant protein $^{\alpha}$thioester derivative [21]. Peptide sequences containing an amino-terminal cysteine residue can then be specifically ligated to the carboxyl termini of these recombinant $^{\alpha}$thioester proteins [24–26], in a procedure termed expressed protein ligation (EPL) or intein-mediated protein ligation (IPL). It is important to note inteins are not the only protein domains that can be used to generate recombinant protein $^{\alpha}$thioesters. Indeed, Beachy and coworkers [27] have demonstrated that the autoprocessing domain of the protein Hedgehog (which is structurally related to inteins) can be used to generate semisynthetic proteins in a manner analogous to EPL.

Methods for generating recombinant amino-terminal cysteine proteins rely on cleavage of an appropriate precursor protein. In the enzymatic method developed by Verdine and coworkers [17], the recognition sequence for the protease factor Xa is introduced immediately in front of the cryptic amino-terminal cysteine in the protein. Because factor Xa cleaves directly after its recognition site, the desired amino-terminal cysteine protein is simply liberated by mild enzymatic treatment. Other proteases that cleave after their recognition site, such as enterokinase or ubiquitin carboxy-terminal hydrolase, should also be compatible with such an approach. An alternative strategy has recently been described that negates the need for this proteolytic step [22,23,28]. This clever approach was again developed from studies on protein splicing, and utilizes inteins that have been engineered to promote direct cleavage at their carboxyl terminus, even with a cysteine residue at the + 1 position. The protein of interest is simply expressed as carboxy-terminal fusion to one of these engineered inteins and the desired material is liberated through spontaneous cleavage at the intein-C-extein junction.

Benzyl $^{\alpha}$thioester containing peptides have been shown to be excellent substrates for the peptide ligase subtiligase [29], a double mutant version of the serine protease subtilisin [29,30]. Welker and Scheraga [31] have recently shown that benzyl $^{\alpha}$thioester derivatives of recombinant proteins can be prepared using intein technology, a method that the authors suggest will allow synthetic peptides to be enzymatically ligated to the carboxyl terminus of recombinant proteins. Although this remains to be experimentally confirmed, this strategy would be a powerful complement to the earlier work of Wells and coworkers [32] who demonstrated that subtiligase can be used to ligate synthetic peptides to the amino terminus of recombinant proteins.

The observation that protein splicing can be triggered by reconstituting inactive amino- and carboxy-terminal fragments of an intein [33–37] provides yet another application of this versatile process in protein engineering. Usually referred to as *trans*-splicing, this phenomenon allows two recombinant proteins to be joined together

in vitro. Each protein is expressed as a fusion with one of a pair of complementary intein fragments; simply combining the two fusion proteins together under appropriate conditions results in a noncovalent association of the intein fragments, activation of protein splicing and so generation of the desired recombinant protein chimera. Most *trans*-splicing studies have used artificially engineered split intein systems [33–35], although a naturally occurring split intein system has recently been observed [36] — a remarkable finding that adds an additional layer of complexity to the post-translational control of protein function. Finally, a semisynthetic *trans*-splicing system has also been reported in which the carboxy-terminal component is a synthetic polypeptide, thereby providing another route to the preparation of semisynthetic proteins [37].

With the availability of the technologies outlined above, all possible permutations for joining recombinant and synthetic polypeptides together are, in principle, amenable through chemical/enzymatic ligation. As illustrated in the following sections, this opens up many exciting opportunities for the application of synthetic chemistry to protein engineering.

Protein–ligand interactions

The ability to perform 'unnatural amino acid mutagenesis' makes the peptide ligation approach well suited for investigating protein–ligand interactions. In a recent example, the interaction between the σ^{70} protein subunit of *Escherichia coli* RNA polymerase and the T4 anti-sigma protein (AsiA) was mapped using expressed protein ligation [25]. A truncated version of the 600 amino acid σ^{70} subunit lacking residues 567–600 was generated as the $^{\alpha}$thioester protein and found to lack AsiA binding activity. A synthetic peptide, corresponding to the missing residues, was then ligated to the carboxyl terminus of the truncated protein, which restored AsiA binding activity, thus elucidating the binding region. This functionally important carboxy-terminal region of σ^{70} can now be readily modified with all manner of biophysical probes and unnatural amino acids through ligation of the appropriate synthetic peptide.

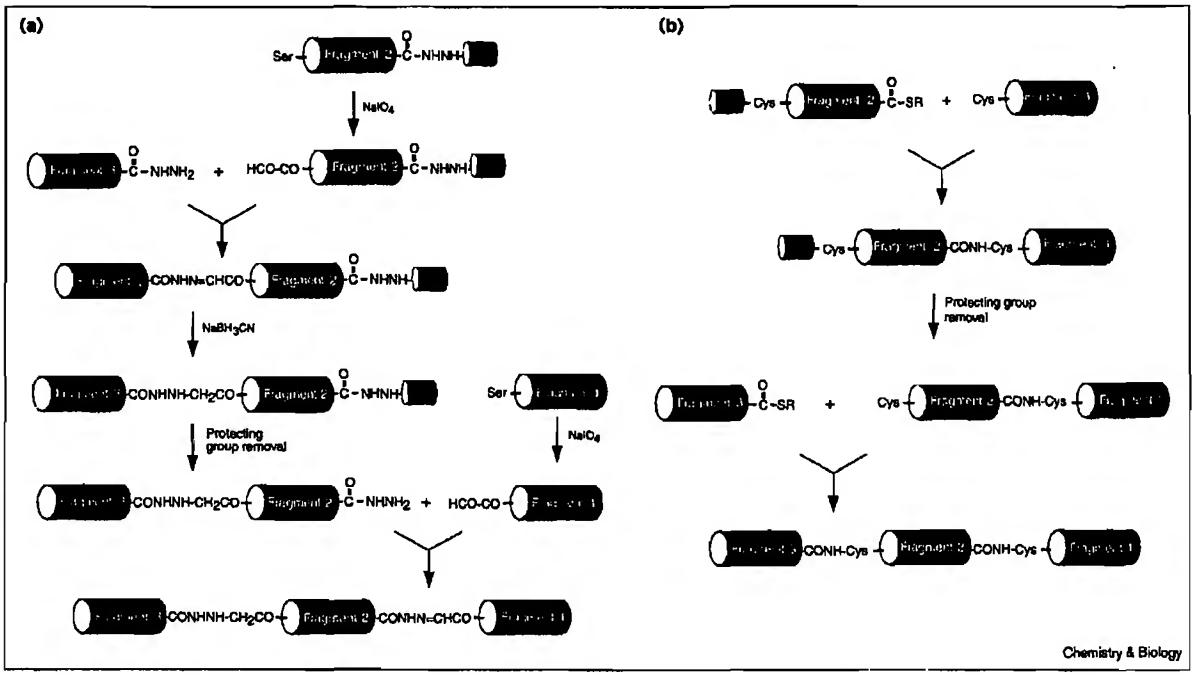
An extension of native chemical ligation has been used to study the interaction between the AP-1 transcription activator complex and its DNA recognition sequence [17,38]. An affinity cleavage reagent was prepared by chemically ligating a synthetic thioester derivative of EDTA to a series of recombinant AP-1 complexes (composed of c-Fos and c-Jun subunits) each possessing an amino-terminal cysteine residue generated by proteolytic cleavage [17]. These semisynthetic proteins have been used in an elegant series of studies to elucidate the structural determinants of orientation within an NFAT•AP-1•DNA ternary complex [17], and to study how this orientation affects transcription activation [38].

An alternative ligation chemistry has been used to specifically attach biophysical probes to recombinant proteins containing an amino-terminal serine residue, which can be converted into a glyoxyl group by mild periodate oxidation [13,39]. This aldehyde functionality can then be chemoselectively reacted with compounds containing either hydrazide, aminooxy or thiosemicarbazide groups to produce the corresponding hydrazone, oxime or thiosemicarbazole derivatives. This approach has been used to generate fluorescein and biotin derivatives of SH3 domains for studying their ligand-binding properties [40,41]. In a seminal series of studies, Proudfoot and coworkers [42] have used these peptide ligation techniques to identify potent inhibitors of HIV-1 infection based on the β-chemokine RANTES. A natural ligand for the HIV-1 coreceptor CCR5, RANTES is a competitive inhibitor of HIV infection *in vitro*, but its pro-inflammatory properties render it of little value as an antiviral agent. Using an oxime ligation approach, these researchers were able to generate an amino-terminally modified version of the protein, AOP-RANTES, which blocks viral infection in a variety of immune cells, but importantly has no pro-inflammatory effects [42]. In a follow up series of studies, native chemical ligation has been used to prepare even more potent RANTES analogs and to facilitate high-resolution structural studies on these molecules [43,44].

The groups of Offord and Rose [13–15] have pioneered the use of the hydrazone- and oxime-forming reactions for chemically ligating synthetic and recombinant peptide fragments together. In one original application, ligation through hydrazone formation was used to investigate the biological activity of a series of granulocyte colony stimulating factor (G-CSF) analogs [13,14]. Recombinant fragments of the protein were first generated through specific proteolysis at in-built Lys–Ser sequences. Amino-terminal aldehyde groups or carboxy-terminal hydrazides were then incorporated into the appropriate fragments through periodate oxidation or reverse proteolysis, respectively. Backbone engineered derivatives of the 174-residue protein were therefore obtained by ligating the fragments back together, and importantly these were shown to retain full biological activity [13]. There followed a sophisticated extension of this work in which a central fragment of G-CSF was replaced by a synthetic peptide, essentially a kind of synthetic cassette mutagenesis [14]. The appropriate reactive functionalities were introduced into the synthetic peptide insert, which then allowed it to be regioselectively reacted with the flanking recombinant pieces of the protein (Figure 2a).

Site-specific modification of peptides and proteins with fluorescent probes offers an extremely powerful way of studying biological processes. In a recent example, a fluorescent analog of the Abelson (Abl) protein tyrosine kinase was generated in which a synthetic dansylated peptide

Figure 2



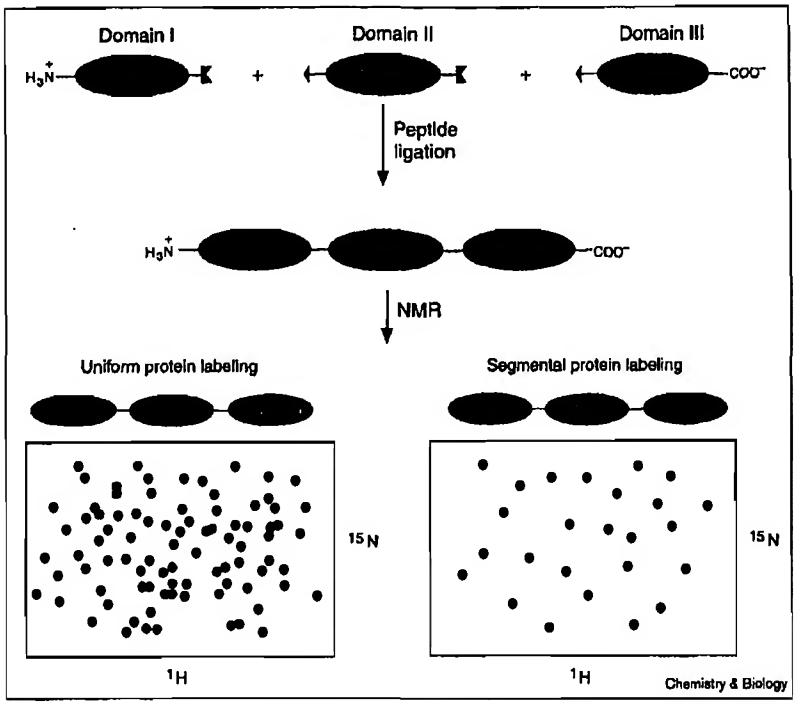
Strategies for the sequential chemical ligation of recombinant and/or synthetic protein fragments utilizing **(a)** the hydrazone bond forming reaction or **(b)** the native chemical ligation reaction. PG refers to a protecting group that can either be chemically or enzymatically removed.

was inserted between the recombinantly derived Src homology 3 (SH3) and SH2 domains of the protein [45]. To perform this synthetic cassette mutagenesis, a sequential native chemical ligation strategy was developed in which all steps were performed at physiological pH (Figure 2b). In this method, the synthetic insert contained a carboxy-terminal thioester and a cryptic amino-terminal cysteine residue masked by a factor Xa cleavable pro-sequence. This reversible cysteine protection was necessary to prevent the insert reacting with itself in either an intermolecular or intramolecular fashion [11,46]. In the first reaction, recombinant amino-terminal cysteine Abl–SH2 (generated by factor Xa treatment of the appropriate precursor) was ligated to the carboxyl terminus of the insert. The pro-sequence was then removed from the ligation product by treatment with factor Xa, to reveal the requisite amino-terminal cysteine for the second ligation reaction. This was facilitated by the addition of recombinant Abl–SH3 $^{\alpha}$thioester (produced using intein technology) to yield the desired insertion product. A series of fluorescence studies indicated that this semisynthetic protein is a specific biosensor for high-affinity bidentate interactions involving the regulatory region of Abl [45].

This significant extension to EPL renders any region of a protein accessible to synthetic manipulation, at least in principle. Moreover, as the factor Xa cleavable pro-sequence can be incorporated at the DNA level, the insert in this strategy can itself be a recombinant protein. Indeed, recombinant sequences that contain both an amino-terminal cysteine and an $^{\alpha}$thioester have been used to produce circular and polymeric proteins [47,48], something previously accomplished using synthetic peptides [11,46]. Two different approaches have been developed, both of which utilize inteins with amino-terminal cleavage activity to produce the $^{\alpha}$thioester moiety, but differ in the way the amino-terminal cysteine is produced. In one report, the amino-terminal cysteine originates from factor Xa proteolysis [47], a strategy that allowed a circular version of an SH3 domain from the protein c-Crk to be efficiently generated and studied. In the so-called TWIN (TWo INtein) approach developed by Xu and coworkers [48], the requisite amino-terminal cysteine was generated using carboxy-terminal intein cleavage, a system that allowed a number of circular and polymeric peptides and proteins to be prepared.

In an indication that peptide ligation could be a useful tool in cell biology, Tam and coworkers [49] used a thiazolidine ligation approach to attach a membrane-permeable peptide sequence (MPS) to a series of bioactive peptides. These researchers ligated a carboxy-terminal aldehyde

Figure 3



Principle of segmental isotopic labeling for structural analysis of proteins using NMR spectroscopy. In the hypothetical example shown, an internal domain of a multidomain protein is selectively labeled with ^{15}N, allowing this region to be selectively analyzed using heteronuclear NMR spectroscopy.

derivative of the MPS to an amino-terminal cysteine peptide and demonstrated that the resulting chimera was imported into eukaryotic cells [49]. The significance of this approach is that it should allow import of both peptide and nonpeptide bioactive molecules into cells.

Structural studies

Peptide ligation is beginning to make important contributions to the field of structural biology, both by providing access to interesting molecules for high-resolution studies and, as will be the primary focus here, through the development of novel strategies that actually aid the structure determination process in nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography [32,50–52].

Although developments over the last several years now allow proteins of up to ~35 kDa to be studied using NMR spectroscopy, larger systems remain inaccessible because of the loss of spectral resolution that occurs as a result of increased line widths and increased numbers of signals with similar chemical shifts. The former of these problems has to some extent been addressed with the development of new methods for spectral observation (reviewed in [53]). Peptide ligation appears to offer a solution to the latter by allowing selected segments of a protein to be isotopically labeled with NMR sensitive nuclei (Figure 3). In principle, segmental isotopic labeling of a large protein will lead to simplified NMR spectra; unlabeled regions of the protein can be filtered out using suitable heteronuclear correlation experiments leaving only signals from the labeled part of the protein. Consequently, segmental labeling should allow NMR structure analysis of discrete regions of very large proteins (Figure 3).

The feasibility of segmental isotopic labeling has recently been demonstrated using two different peptide ligation strategies—*trans*-splicing [50] and expressed protein ligation [51]. Yamazaki and coworkers used a *trans*-splicing system based on the PI-*Pfu1* intein to selectively ^{15}N label the carboxy-terminal domain of the *E. coli* RNA polymerase α subunit [50]. The carboxy-terminal domain of α (αC) was expressed as two fragments, each attached to one half of the split intein system. Reassembly of the intein module induced protein splicing, resulting in the generation of the intact αC domain. Each half of the protein was selectively labeled by simply expressing the corresponding split intein fragment in ^{15}N-enriched medium. Heteronuclear NMR experiments on these samples clearly revealed the improvement in spectral resolution that segmental labeling provides [50]. In a complementary study, expressed protein ligation was used to ^{15}N label a single domain within a Src-homology domain pair

derived from the Abl protein tyrosine kinase [51]. An ethyl $^{\alpha}$thioester derivative of recombinant Abl–SH3 was generated from the corresponding intein fusion and then chemically ligated, under physiological conditions, to ^{15}N-labeled recombinant Abl–SH2 possessing a factor Xa generated amino-terminal cysteine. Comparison between the ^{1}H{^{15}N} NMR spectra of fully labeled and segmental labeled Abl–SH(32) again illustrated the power of segmental isotopic labeling for studying large proteins using NMR spectroscopy.

Peptide ligation also provides a route to protein derivatives for use in X-ray structure determination. In an early example, Wells and coworkers [32] used subtiligase-mediated peptide ligation to attach a mercurated-cysteine-containing peptide to human growth hormone. The ability to incorporate heavy-atom-laden sequences into proteins using ligation is likely to prove useful for solving the relative phases in protein crystal structures using isomorphous replacement techniques. In a similar vein, peptide ligation also provides an alternative route to selenomethionine-containing proteins, which are now commonly used for solving the phase problem in conjunction with multiwavelength anomalous diffraction (MAD) [54]. The feasibility of this strategy was recently demonstrated by Lolis and coworkers [52] who used native chemical ligation to prepare a specific selenomethionine derivative of the chemokine MIP-II protein for subsequent crystallographic studies.

Enzymology

A number of enzyme-catalyzed reactions have been probed using peptide-ligation approaches. Fine details of enzyme mechanism can be directly studied through the incorporation of unnatural amino acids that have unique chemical properties into an enzyme active site. This approach is exemplified by a series of studies on the HIV-1 aspartyl-protease, a 99-residue polypeptide that is active as the homodimer. In a pioneering peptide-ligation study, the fully active protease was produced by chemical ligation of two unprotected synthetic fragments, each of ~50 amino acids in length [55]. Synthetic access has allowed the covalent structure of the enzyme to be manipulated in ways possible only through chemical synthesis, by modifying the sidechains or backbone of the protein [56,57], or incorporating NMR probe nuclei in the active site [58], which has allowed fundamental mechanistic questions to be asked. Native chemical ligation has been used to study intermolecular interactions between various serine proteinases and the protein inhibitors OMKTY3 [59] and eglin c [60]. Synthetic access to these protein inhibitors has allowed a systematic and quantitative analysis of the contribution of intermolecular backbone–backbone hydrogen bonding to the stability of an enzyme–inhibitor complex. In the recent study on eglin c, a series of five protein analogs were prepared, each containing a unique mainchain -NHCO- to -COO- modification and designed to delete a particular hydrogen-bonding interaction with the proteinase [60]. Using these synthetic proteins, the authors were able to show that the contribution of hydrogen-bonding to stability is highly context dependent and that there is a complex reciprocity between rigidity and adaptability in determining inhibition.

In a beautiful piece of protein engineering, the 124-residue enzyme ribonuclease A was assembled from six synthetic fragments using the subtiligase technology described earlier [30]. The catalytic histidine residues at positions 12 and 119 were then replaced by 4-fluorohistidine by modifying the corresponding synthetic peptide. The difference in pKa between this fluoro derivative and the natural histidine residue was used, among other things, to fully elucidate the general acid/general base mechanism employed by the enzyme.

Intein technology is also beginning to see application in enzymology. In one recent example, the role of the carboxy-terminal carboxyl group of T7 RNA polymerase in catalysis was investigated [61]. The protein was expressed as an amino-terminal fusion to an intein and then cleaved with either cysteine, cysteine methyl ester or 2-mercaptoethylamine to generate the corresponding carboxy-terminal cysteine, cysteine methyl ester or decarboxy-cysteine derivatives. Study of these semisynthetic analogs revealed a direct role for the carboxy-terminal carboxylate group in Mg^{2+}-dependent catalytic activity. In a second example, Begley and coworkers [62] demonstrated that recombinant protein $^{\alpha}$thiocarboxylates can be prepared from the corresponding intein fusions—a process that was developed to study the enzymology of thiamin biosynthesis, which involves a protein thiocarboxylate intermediate.

Post-translational modifications

The difficulty in producing proteins that have defined and specific patterns of post-translational modification has hindered studies on their functional role. This is an area in which peptide ligation could have a major impact, considering these alterations are central to the molecular events controlling cellular signaling networks and nature's method for conferring functional diversity onto the same translated sequence. A typical example is the regulation of the Src family of protein tyrosine kinases through tyrosine phosphorylation of their carboxy-terminal tails. This post-translational event induces an intramolecular association between the tail region and an SH2 domain, which acts to down regulate kinase activity. This tyrosine phosphorylation reaction is catalyzed by the kinase Csk, which is highly homologous to Src family members but, interestingly, lacks this regulatory tail region. Peptide ligation was used to test the hypothesis that addition of a phosphotyrosine tail to Csk would result in down-regulation of its kinase activity, in a manner analogous to Src kinases [24]. Full length Csk (450 amino

acids) and a short synthetic phosphopeptide were hooked together using EPL to give the ~53 kDa phosphoprotein. The semisynthetic Csk protein did indeed form the expected intramolecular phosphotyrosine–SH2 association but surprisingly showed an increase in the catalytic activity relative to the wild-type protein.

Systematic studies on glycoproteins have proved troublesome because of difficulties in producing homogeneous samples of defined oligosaccharide structure. Consequently, a number of synthetic methods have been developed to address this problem, including chemical and enzymatic ligation strategies (reviewed in [63]). The general approaches developed by Bertozzi and coworkers make use of the ability to incorporate unique ketone or aldehyde groups into peptides [64,65] and glycopeptides [66], respectively. Such groups can then be elaborated in a truly chemoselective fashion through oxime/hydrazone/thiosemicarbazole chemistries to give the desired neoglycopeptides [64–66]. A related strategy has been developed in which free reducing sugars are chemoselectively reacted with peptides bearing aminooxy groups [67–69]. Indeed, Mutter and coworkers [69] demonstrated that the use of *N,O*-disubstituted aminooxy groups allowed synthetic peptides to be glycosylated with high anomeric stereoselectivity.

Chemical methods are now available for the preparation of phosphorylated, glycosylated and prenylated versions of small synthetic peptides. This, coupled with the availability of the peptide-ligation technologies discussed above, suggests that it should be possible to assemble large proteins that have defined post-translational modifications. Indeed, a chemoenzymatic ligation approach has recently been used to prepare unique glycoforms of the enzyme RNAse B from constituent peptide fragments [70]. It would therefore appear that synthetic chemistry is in an excellent position to drive fundamental advances in this important area of protein biology.

Future outlook

The peptide-ligation field has evolved rapidly over the last several years. This has brought us to the point where peptide building blocks can be efficiently ligated together using any one of a number of technologies. Moreover, as we have attempted to illustrate in this review, synthetic and recombinant polypeptides can now be freely intermixed in these ligation strategies, allowing extremely large semisynthetic proteins to be manipulated using the tools of organic chemistry. There are still several technical problems, however, that need to be addressed, particularly in the native chemical ligation strategy, which is arguably the most powerful peptide-ligation approach. One important challenge for the future will be to develop 'chemical tricks' that negate the need for an amino-terminal cysteine residue in one of the peptide segments. Possible solutions to this problem might involve the use of transient auxiliary groups [71] or the use of conformationally-assisted ligations [72]. The field would also benefit greatly from the development of reliable routes to peptide $^{\alpha}$thioesters using the Fmoc–SPPS strategy — the method of choice for synthesizing phosphopeptides. Recent developments in resin-linker technology suggest this problem will be short lived [73–75].

The ability to perform sequential ligation reactions is extremely exciting because it provides synthetic access to very large proteins and, when interfaced with expressed protein ligation, allows the segmental isotopic labeling of internal regions of a protein. As pointed out earlier, these sequential ligation strategies are technically demanding to perform, and therefore the routine application of this approach will require new developments. Perhaps the most likely solution to this problem will involve performing stepwise ligations on a solid support (akin to SPPS), something that has already been formally achieved using synthetic peptides [10,11,76] and, in our own group, using a combination of synthetic and recombinant peptides (G.J.C. and T.W.M. unpublished observations).

This review began by pointing out the enormous opportunities that the proteome project presents to chemists. The field of peptide ligation is sure to play an expanding role in this endeavor by providing rapid access to novel sequences through total synthesis and by facilitating new lines of investigation in the areas ranging from structural biology and biochemistry to basic cell biology.

Acknowledgements

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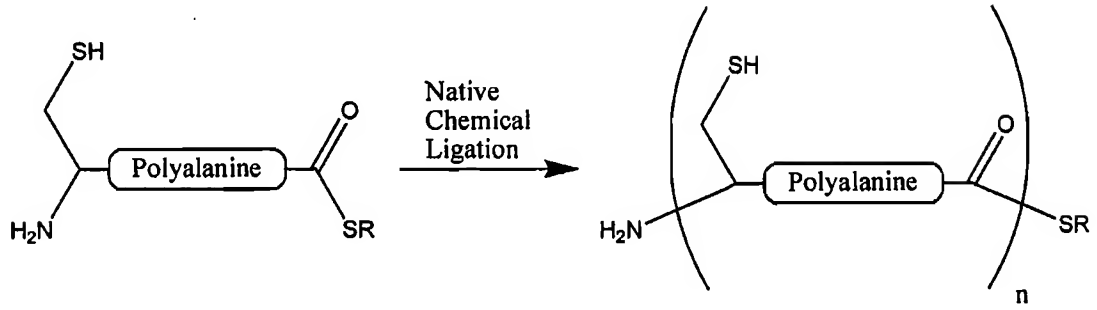
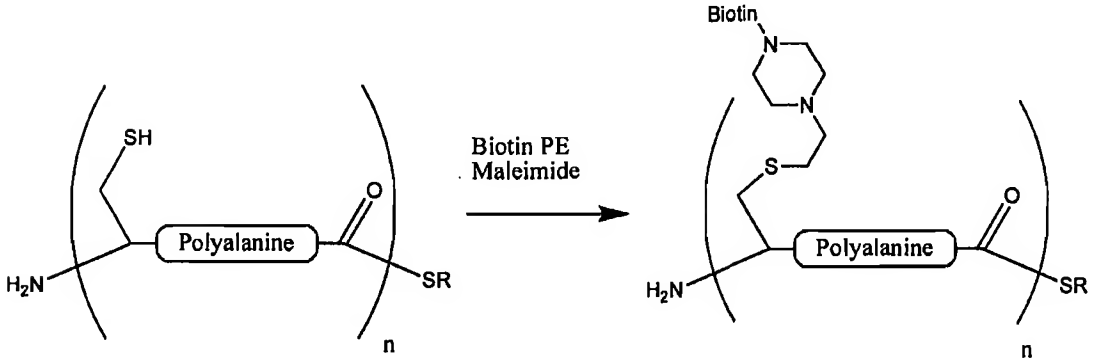
EXHIBIT B

Figure 1: Preparation of Ferritin-Derived Polypeptide

Step 1:



Step 2:



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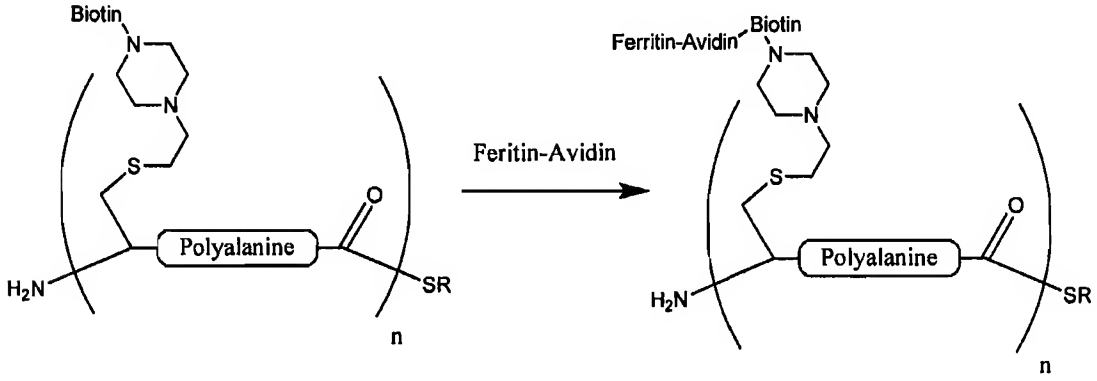

EXHIBIT C

review

CLIN. CHEM. 37/5, 625-636 (1991)

The Biotin–(Strept)Avidin System: Principles and Applications in Biotechnology

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The biotin–(strept)avidin system has been used for many years in a variety of different applications. Here we present a general overview of the system, describe its components and advantages, and show how the system is used in various applications, with emphasis on immunological and nucleic acid hybridization assays. This system is now considered a versatile independent technology with broad applications in many branches of biotechnology. Clearly, its use will continue to grow in the years to come.

Additional Keyphrases: *nonisotopic immunoassay · nucleic acid hybridization · affinity chromatography · labeling techniques · DNA probes*

Living organisms usually develop highly specific defense mechanisms, which help them survive in competitive and unfriendly environments. Scientists sometimes unravel such defense mechanisms and use them in vitro, in a completely different context. For example, bacterial restriction endonuclease enzymes revolutionized the field of molecular cloning when molecular biologists recognized in them a cheap, easy, and highly specific and versatile way of cutting nucleic acids. Here we will discuss the utility of avidin, a protein found in egg white, and streptavidin, a similar protein found in *Streptomyces avidinii*, which have the ability to bind with very high affinity the vitamin biotin (*1*). This interaction is thought to represent a natural defense mechanism because the binding with avidin or streptavidin of biotinylated enzymes that participate in CO_2 transfer inactivates the enzymes and thus inhibits the growth of bacteria that depend on biotinylated enzymes.

Although many other ligand–binder interactions are described in the literature (*2*), the biotin–avidin or biotin–streptavidin interaction has some unique characteristics that make it ideal as a general bridge system in many diverse applications:

(*a*) The noncovalent interaction of avidin or streptavidin with biotin is characterized by a formation (affinity) constant of 10^{15} L · mol^{-1}. This is among the highest formation constants reported, about 10^3–10^6 times greater than for the interaction of ligands with their specific antibodies. This high affinity ensures that, once formed, the complex is not disturbed by changes in pH, the presence of chaotropes, or manipulations such as multiple washings when the complex is immobilized.

(*b*) Avidin or streptavidin binding to biotin is specific enough to ensure that the binding is directed only to the target of interest.

(*c*) Both streptavidin and avidin possess four binding sites per molecule. This very useful property makes it possible to use multiply biotinylated moieties (e.g., polybiotinylated enzymes) and avidin or streptavidin to create mixtures consisting of polymers of biotinylated moieties with avidin or streptavidin (*3*). These polymers could still have some free binding sites for biotin, thus becoming more-sensitive detection reagents in pertinent applications.

(*d*) Biotin is a small molecule (244.31 Da) that, when introduced into biologically active macromolecules, in most cases does not affect their biological activity, e.g., enzymic catalysis or antibody binding. Moreover, using biotin to derivatize small molecules (e.g., mononucleotides or thyroid or steroid hormones) yields biotinylated moieties that still can act as enzyme substrates or are able to bind specific antibodies. Thus, biotinylation does not usually alter many properties of the molecules.

(*e*) On many occasions, avidin or streptavidin must be chemically derivatized with various organic reagents for conjugation with low- or high-M_r compounds or solid supports. As a rule, both streptavidin and avidin are exceptionally stable molecules and their biotin-binding activity can survive harsh reaction conditions and extensive derivatizations (*4*).

In the biotin–avidin or biotin–streptavidin system, one participating component must always be biotinylated (Table 1). The various biotinylation strategies and chemical reactions will be mentioned in detail below. Fortunately, many biotinylation reagents are now commercially available; these are extremely easy to use by following well-established procedures available in the literature and from the reagent manufacturers. Also, highly purified avidin and streptavidin are commercially available and they are relatively cheap. Large collections of derivatized avidin and streptavidin—e.g., with fluorophores, enzymes, metals, proteins, solid phases—are also commercially available (Table 2). These reagents, in combination with biotinylated moieties (Table 1), have contributed in the quick dissemination of diverse applications and encouraged many investigators to use the system.

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Table 1. Selected List of Biotinylated Molecules and Their Possible Applications

Biotinylated moiety	Possible applications
Anti-immunoglobulins, Protein A, Protein G	Immunological assays, flow cytometry, cell sorting, immunohistochemistry, Western blots
Lectins	Glycoconjugate studies, mitogenic stimulation studies
Anti-lectins	Localization of lectin receptors
Enzymes (ALP, β-galactosidase, glucose oxidase, HRP)	Immunological assays, nucleic acid hybridization
Ferritin, hemocyanin	Electron microscopy
Agarose, cellulose	Affinity chromatography
Anti-avidin, anti-streptavidin	Amplification assays
Nucleotides	Nucleic acid hybridization
DNA	Nucleic acid hybridization, molecular mass markers, DNA sequencing
Hormones	Affinity chromatography, receptor–ligand interaction studies
Cells	Hybridoma production

ALP, alkaline phosphatase; HRP, horseradish peroxidase.

Table 2. Selected List of Avidin or Streptavidin Conjugates and Their Possible Applications

Conjugate of avidin or streptavidin with	Possible applications
Enzymes (ALP, HRP, β-galactosidase, glucose oxidase, etc), fluorophores (fluorescein, coumarins, rhodamines, phycoerythrin, Texas Red), Eu^{3+}, other metals	Immunological assays, flow cytometry, cell sorting, immunohistochemistry, Western blots, nucleic acid hybridization
Eu^{3+}-chelates[a]	Immunological assays, Western blots, nucleic acid hybridization
Ferritin, gold	Electron microscopy
Chemiluminescent labels	Immunological assays, Western blots, nucleic acid hybridization
Agarose	Affinity chromatography
Magnetic particles	Nucleic acid hybridization, affinity chromatography, DNA sequencing
Polystyrene	Immunological assays

[a] With 4,7-bis-chlorosulfophenyl-1,10-phenanthroline-2,9-dicarboxylic acid. Abbreviations as in Table 1.

The literature on the system has exploded during the last 10 years, and many specialized reviews have been published (*5–11*). A comprehensive volume of *Methods in Enzymology* is devoted to the avidin–biotin technology (*12*) and can serve as a reference for interested readers and experimentalists. Given the special interests of the readers of *Clinical Chemistry*, we will cover, with some detail, applications of the biotin–avidin system in immunochemical and nucleic acid hybridization techniques. Other applications will also be mentioned.

Avidin and Streptavidin

Avidin, a 67-kDa glycoprotein, consists of four identical subunits of 128 amino acids each. The amino acid sequence of the subunits is known (*13*). Recently, Gope et al. cloned the avidin gene from chicken oviduct (*14*). The cloned gene was successfully expressed in *Escherichia coli*, providing biologically active recombinant avidin.

In theory, avidin could be purified by adsorption on an insoluble biotinylated matrix; however, because of the very high affinity of the biotin–avidin complex, the avidin is not easily eluted. Today, affinity-purified avidin can be prepared by using insoluble matrices, e.g., Sepharose covalently linked with iminobiotin (*15–19*). Iminobiotin is a biotin derivative (Figure 1) containing a guanidinium group instead of a ureido group. When the guanidinium group is not protonated, i.e., is at relatively high pH (e.g., 11.0), iminobiotin binds strongly to avidin, although with ~100-fold less affinity than biotin. At low pH (e.g., 4.0), the guanidinium group is protonated, and imidobiotin does not bind to avidin. Thus, avidin can be specifically adsorbed on an iminobiotin–Sepharose matrix at pH 11.0 and then eluted in pure form at pH 4.0.

Avidin is a glycoprotein containing both mannose and *N*-acetylglucosamine (*13*) and has an isoelectric point (pI) of ~10 (*2*). The major problem of using avidin in some applications is the high nonspecific binding, which is attributed to both the presence of the sugars and the high pI. Nonglycosylated avidin has essentially the same biotin-binding characteristics as glycosylated avidin and can be isolated from crude avidin preparations (~30% of total) by using concanavalin-A affinity columns that adsorb only the glycosylated fraction (*20, 21*). Alternatively, deglycosylation by enzymatic cleavage could be used. Deglycosylated avidins are expected to have lower nonspecific binding. Commercial manufac-

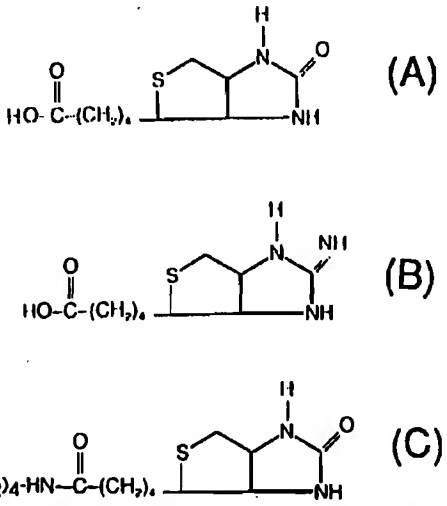

Fig. 1. Chemical structures of biotin (*A*), iminobiotin (*B*), and biocytin (*C*)

turers are using proprietary procedures to produce low-nonspecific-binding avidins suitable for specialized applications, e.g., avidin-D and avidin-DN (Vector Laboratories, Burlingame, CA).

The biotin-binding protein streptavidin also consists of four subunits but contains no carbohydrate; its pI is 5–6. Streptavidin is widely used in place of avidin because of its lower nonspecific binding. This protein is isolated from the culture broth of *S. avidinii* by ammonium sulfate precipitation, ion-exchange chromatography, and crystallization. Alternatively, iminobiotin columns can be used directly with the bacterial broth.

The streptavidin gene has recently been cloned and sequenced (*22*). From the complete nucleotide sequence, the amino acid sequence was deduced and compared with that of avidin. Many regions of the two molecules showed significant homology. The molecular mass of streptavidin was previously reported to be 60 kDa for the product isolated as described above by an initial ammonium sulfate precipitation (many commercially available products are prepared with such methods). From the gene sequence, a mass of 66 kDa was predicted; a 75-kDa product was isolated on iminobiotin columns. It is now known that native streptavidin of relatively high mass (66–75 kDa) can be converted to a lower-mass form (i.e., 60 kDa) by proteolytic digestion at both the N and C termini, which occurs during the ammonium sulfate precipitation step; this conversion is prevented if an iminobiotin column is used (*18*). The lower-M_r streptavidin is the commercially available product (called "core" or "truncated" streptavidin), which has better biotin-binding characteristics than the native protein. Native streptavidin can be converted to core streptavidin by a simple digestion with proteinase K (*23*).

Biotin

Biotin (Figure 1), also known as vitamin H, is present in minute amounts in every living cell. It acts as a co-factor of carboxylating enzymes, e.g., pyruvate carboxylase, which catalyzes the formation of oxaloacetate by condensation of pyruvate and CO_2. The carboxyl group of biotin is the site of attachment of the molecule to ε-amino groups of lysine residues through an amide bond. During a carboxylation reaction, the CO_2 is first fixed to the iminogroup of biotin to form carboxy-biotin, which in a second step passes CO_2 to the substrate, e.g., pyruvate.

Numerous biotin derivatives are available and will be discussed in a separate section. Biocytin, an adduct of biotin and lysine (*N*-ε-biotinyl-L-lysine), is found naturally but also is synthesized from biotin and lysine. Biocytin derivatives prepared for labeling are mentioned below.

Biotinylation Procedures

One of the basic components in a biotin–avidin-based system is the biotinylated moiety, which can be a protein, a polysaccharide, a nucleic acid, a low-M_r substance, etc. To biotinylate such diverse classes of compounds, researchers have developed several different biotinylation reagents (active biotin derivatives). In addition, other biotinylation reagents have been developed for specific applications: e.g., cleavable reagents that can be used for biotinylation with the option of removing biotin at a later stage by reduction of a disulfide bond or hydrolysis of phenyl ester linkages (*24–27*).

Table 3. Major Biotin Labeling Strategies for Proteins and Carbohydrates

Molecule for biotinylation	Reactive group or moiety	Biotinylation reagent
Proteins, haptens, or peptides	Amine	NHS-biotin, NHS-LC-biotin
	Tyrosyl, histidyl	*p*-Diazobenzoylbiocytin
	Sulfhydryl	3-(*N*-Maleimidopropionyl) biocytin or Iodoacetyl-LC-biotin
	Carboxyl	Carbodiimide and biotin hydrazide
Glycoconjugates, carbohydrates (including antibodies and ribonucleotides)	Vicinal hydroxyls	Periodate oxidation and biotin hydrazide

An exhaustive review of many different biotinylation reagents has been published recently (*28*). Below, we describe biotinylation procedures for proteins, sugars, and nucleic acids. However, the same procedures can be used for other compounds bearing reactive groups. These procedures are summarized in Tables 3 and 4.

Proteins

In the vast majority of cases, proteins are biotinylated via the ε-amino groups of lysines by using an *N*-hydroxysuccinimide ester (NHS-ester) of a biotin analog (*29*).[3] Figure 2 shows the two most commonly used reagents, along with a typical biotinylation reaction. The more recently developed reagent, NHS-LC-biotin, is the reagent of choice: it decreases steric hindrance because it has an extra spacer arm between biotin and protein. Consequently, the biotin is more exposed from the protein surface and is more available to bind avidin or streptavidin.

Biotinylation reactions with NHS-biotin derivatives (available commercially from many companies) are simple to perform. We use a reaction pH of 9.1 adjusted with 0.25–0.50 mol/L carbonate buffer. Proteins are used at a concentration of 0.1–2 g/L and, before biotinylation, are extensively dialyzed in 0.1 mol/L bicarbonate solution, to remove any interfering small molecules (e.g., amine-containing buffers). Biotin analogs, dissolved in a minimum volume of dimethyl sulfoxide, are added at 50- to

[3]Nonstandard abbreviations: NHS, *N*-hydroxysuccinimide; LC, long chain; dNTP, deoxynucleotide triphosphates; TdT, terminal deoxynucleotidyl transferase; PCR, polymerase chain reaction; AMPPD, 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane; NBT, 4-nitroblue tetrazolium; and BCIP, 5-bromo-4-chloro-3-indolyl phosphate.

Table 4. Biotinylation of Nucleic Acids

Method	Labeled nucleic acid	Principle
Nick translation	dsDNA	Enzymatic: DNAse I and DNA polymerase I with biotinylated dNTP or their biotinylable precursors
Random priming	dsDNA	Enzymatic: Klenow enzyme, random sequence deoxyribonucleotides and biotinylated dNTP or their biotinylable precursors
Transcription	RNA	Enzymatic: RNA polymerase and biotinylated NTP with dsDNA template
Photobiotin	dsDNA, ssDNA, RNA	Chemical bonding of photobiotin to nucleic acid
Transamination	ssDNA, dsDNA	Generation of biotinylable N^4-substituted cytosines via sodium bisulfite and ethylenediamine
Psoralen derivatives	dsDNA with unlabeled ss regions	Intercalation of psoralen into dsDNA and interstrand link after irradiation
3'-end tailing	3'-end-labeled ssDNA or dsDNA	Enzymatic: TdT in the presence of biotinylated dNTP or biotinylable dNTP
Periodate oxidation	RNA	As in Fig. 3
T4 RNA ligase	RNA	Enzymatic 3'-end labeling with T4 RNA ligase and biotinylated ADP-derivatives
Linker or deoxythimidine phosphoramidite	Short 5'-end labeled ss synthetic oligonucleotide	Introduction of aliphatic amino group at 5'-end during automated phosphoramidite synthesis
Biotinylated nucleoside phosphoramidite	Short ss synthetic oligonucleotides labeled at any position	Introduction of biotinylated bases at any position during automated phosphoramidite synthesis
Nonnucleosidic biotinylated linker phosphoramidite	Short ss synthetic oligonucleotides biotinylated singly or multiply at their 5'-ends	Single or multiple introduction of nonnucleosidic biotinylated linker at 5'-end during automated phosphoramidite synthesis
PCR	dsDNA	Enzymatic incorporation of biotinylated nucleotides by Taq polymerase during PCR

ds, double-stranded; ss, single-stranded.

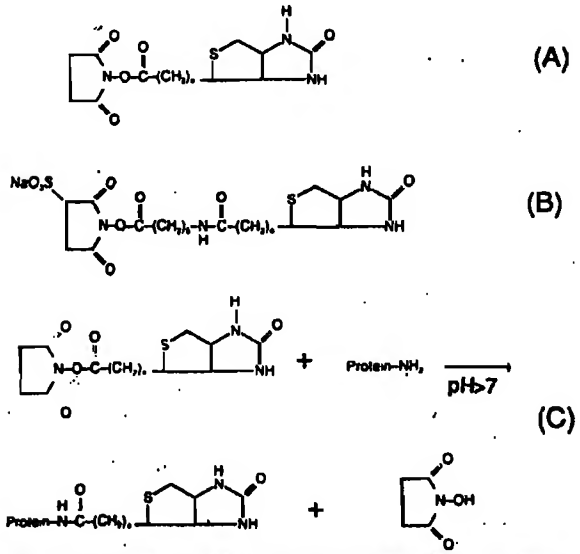

Fig. 2. Chemical structures of NHS-biotin (*A*) and NHS-LC-biotin (*B*)
(*C*) A biotinylation reaction (protein + NHS-biotin). Multiple biotinylation occurs when the protein bears more than one $-NH_2$ group. The size of the molecules is not shown to scale

500-fold molar excess in relation to the protein concentration and incubated at room temperature for 1 h. The mixture is then used either as is or dialyzed against a 0.1 mol/L $NaHCO_3$ solution to remove unreacted biotin.

As is well documented, excessive biotinylation may inactivate a biologically active molecule. To avoid this, it is a good practice to perform trial biotinylations with various amounts of excess biotin reagent to determine optimal performance. The extent of biotinylation does not usually need to be calculated; however, there are methods for doing so, and they are described in reference *30*.

Alternative protein-biotinylation procedures are based on the targeting of groups other than the ϵ-amino groups of lysine residues. The reagent *p*-diazobenzoylbiocytin is specific for tyrosyl and histidyl amino acid side chains. Proteins that contain free -SH groups can be biotinylated by using 3-(*N*-maleimidopropionyl) biocytin or iodoacetyl-LC-biotin. The same reagents can be used with proteins whose S-S groups are reduced to -SH or proteins that are first thiolated. Carboxyl groups of proteins can also be used for biotinylation with biocytin hydrazide and a water-soluble carbodiimide reaction. Glycoproteins can be biotinylated through their sugar moieties by using biotin hydrazide or biotin-LC-hydrazide, as discussed further in the next section.

Carbohydrates

Carbohydrates, glycoproteins, or other glycoconjugates are easily biotinylated by using biotin-LC-hydrazide or biocytin hydrazide (*31–33*) (Figure 3). The glycoconjugate is first reacted with $NaIO_4$ to oxidize vicinal hydroxyl groups of the sugars to aldehyde groups. These groups are then allowed to react with biotin hydrazide, biotin-LC-hydrazide, or biocytin hydrazide to produce the biotinylated sugar moiety. This procedure has been used only occasionally to biotinylate antibodies. One group (*26*) found that this procedure gave inferior results in comparison with biotinylating

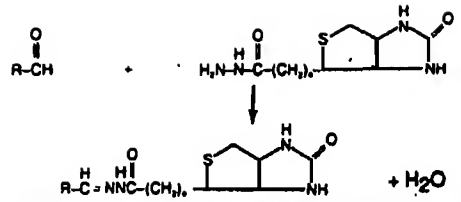

Fig. 3. Reaction of an aldehyde, generated from the oxidation of a *cis*-diol by $NaIO_4$, with biotin hydrazide

antibodies through the ε-amino groups. In our hands, biotinylation of antibodies with NHS-LC-biotin yielded a twofold greater signal in time-resolved fluorometric immunoassays involving labeled streptavidin than did the same antibodies labeled via sugar moieties with biotin-LC-hydrazide. In addition, in biotinylation of other glycoproteins, e.g., bovine thyroglobulin, we have found that, during the $NaIO_4$ oxidation step, the aldehyde groups generated react with amino groups of the protein, resulting in protein polymerization.

Nucleic Acids

Nucleic acid biotinylation can be accomplished with several different procedures, most of which have evolved very recently. In general, cloned nucleotide fragments are biotinylated with different strategies from those used with small synthetic oligonucleotides. The major interest in biotinylating nucleotides arises mainly from the need to devise highly sensitive nonisotopic hybridization assays.

Many procedures for nucleic acid biotinylation are based on enzymatic catalysis; others are chemical methods. Enzymatic biotinylation procedures rely on the availability of biotinylated nucleotide analogs that can act as enzyme substrates. Alternatively, nonbiotinylated nucleotide derivatives can be used as substrates but, after they are incorporated into DNA, they can be chemically biotinylated because they carry into their structure an easily biotinylable group (e.g., an aliphatic primary amine group).

The first reported biotinylated nucleotides were biotinylated uridine triphosphate (UTP) and deoxyuridine triphosphate (dUTP) (Figure 4) (*34*, *35*). Biotin is attached to the 5-position of uridine base through an allylamine linker. The distance of biotin from the nucleotide base, measured in numbers of atoms, is shown in the name of the molecule, as biotin-n-base, e.g., biotin-11-dUTP and biotin-16-dUTP. More recently, biotinylated dATP and dCTP with linkers of between three and 17 atoms long have been described (*36*). Precursors of these molecules are modified bases that contain linker arms with free $-NH_2$ groups, e.g., N^6-(6-aminohexyl) dATP (Figure 4), and are thus easily biotinylated with NHS-LC-biotin. Many biotinylated nucleotides and some of their precursors are commercially available. Another useful class of biotinylated nucleotides consists of nucleotides that contain the S-S group within the structure of the linker arm. These "releasable" nucleotide analogs can be used in applications where the

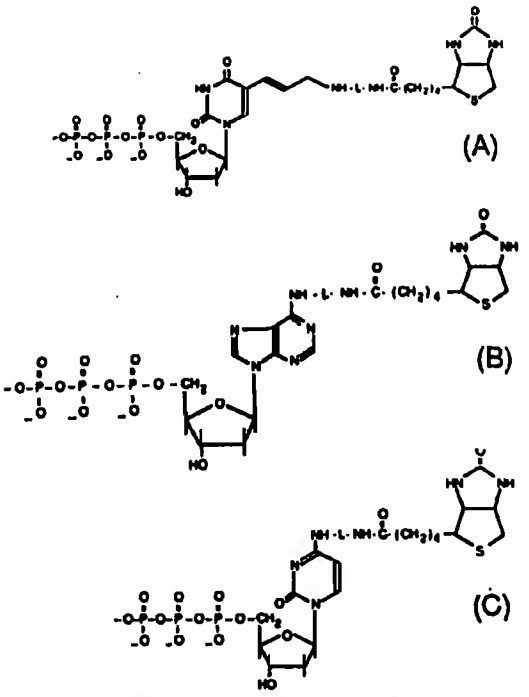

Fig. 4. Biotinylated deoxynucleotide triphosphates

(*A*) dUTP derivatives. *L* can be a variable structure; e.g., $L = CO(CH_2)_5$ will yield biotin-11-dUTP, and $L = CO(CH_2)_2SS(CH_2)_2$ will yield biotin-12-SS-dUTP. (*B*) dATP derivatives. $L = (CH_2)_6$ yields biotin-7-dATP. When $L = (CH_2)_6NH_2$, a biotinylable precursor of dATP is generated. (*C*) dCTP derivatives. $L = (CH_2)_6$ yields biotin-7-dCTP. Adapted by permission from ref. *12*

nucleic acid needs to be released after its binding to streptavidin (*25*, *37*).

The biotinylated nucleotide analogs or their precursors, e.g., N^6-(6-aminohexyl) dATP, can be introduced into DNA by using two general and well-established enzymatic procedures: nick translation and random priming. In nick translation, relatively large (>1 kb) double-stranded DNA is allowed to be attacked by the enzyme DNAse I, which creates "nicks" (holes) at random points of one DNA strand. A second enzyme in the mixture, *E. coli* DNA polymerase I, repairs the nicks by both its 5′ → 3′ exonuclease activity (to chew off more nucleotides) and its 5′ → 3′ polymerase activity (to fill in the gap). During the 5′ → 3′ polymerization, the biotinylated nucleotides or precursors in the reaction mixture are incorporated into the DNA. Although in theory one can incorporate more biotins by using only biotinylated dNTP in the reaction mixture, this is not usually attempted for two reasons: the highly biotinylated DNA may not be suitable to use as a probe (because it may not reassociate with the target with acceptable specificity, affinity, and kinetics), and incorporation of >40 biotinylated nucleotides per kilobase of DNA does not improve the sensitivity of the assays developed (probably because of steric hindrance problems associated with streptavidin binding) (*36*). We have also seen this effect with highly biotinylated proteins, e.g., antibodies labeled with >20 biotins. Nick translation not only labels

but also fragments the original DNA. The ratio of the enzymes DNase I and DNA polymerase I and the incubation time and temperature of the reaction are adjusted so that the labeled probes are 500–1500 bases long. Nick translation kits are commercially available from several manufacturers, making the procedure easy and reliable to apply even by nonspecialists. In these kits, usually one biotinylated nucleotide (e.g., biotin-7-dATP) is incorporated into the reaction mixture along with three unlabeled nucleotides.

The random priming method was originally developed to label DNA radioactively (*38*); it can be used with probes <500 bp long. The method is based on the denaturation of DNA by heating and the subsequent annealing to the DNA, upon cooling, of random-sequence oligodeoxyribonucleotides. The DNA-random oligonucleotide hybrids are substrates for the Klenow fragment of *E. coli* DNA polymerase I, which extends in the 5′ → 3′ direction, incorporating nucleotides and any biotinylated nucleotides in the newly synthesized strands. Again, random priming kits are commercially available and are extremely easy to use. Protocols involving biotinylated nucleotides (e.g., biotin-11-dUTP, which is incorporated as if it were dTTP, or biotin-7-dATP) are also available from these manufacturers.

In any case involving a precursor of biotinylated nucleotides, e.g., N^6-(6-aminohexyl) dATP, the product can be easily biotinylated through use of NHS-LC-biotin.

Cloned double-stranded probes inserted into transcription vectors that contain the promoters for SP6 and T7 RNA polymerase can be transcribed in the presence of biotinylated ribonucleotide triphosphates to produce single-stranded biotinylated riboprobes.

Labeling of DNA can also be achieved by other procedures. A new biotinylation reagent, photobiotin (Figure 5) (*39*), is now commercially available and has been used to label not only DNA but also proteins and RNA (*40*). Photobiotin contains the biotin moiety attached to a photoactive aryl azide group through a linker. Upon exposure to intense visible light (350–370 nm) for 15–20 min, the aryl azide group is converted to a highly reactive aryl nitrene, which reacts and biotinylates the DNA. The biotin incorporation is about 5–10 biotins per kilobase of DNA. The bond is very stable, although its exact nature is not known.

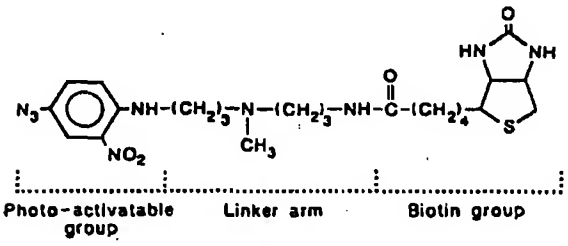

Fig. 5. Chemical structure of photobiotin
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Another method used to label DNA chemically is based on the well-known transamination reaction of cytosine residues with sodium bisulfite and a diaminoalkane, e.g., ethylenediamine (Figure 6) (*41*). This reaction works with single-stranded DNA (prepared by heating double-stranded DNA at 100 °C for 3 min) in one step in the presence of both bisulfite and ethylenediamine. The end product (N^4-substituted cytosine) can then be easily biotinylated by using an NHS-ester of biotin (see Figure 2). The biotin incorporation with this method can be as great as 30–100 biotins per kilobase of DNA and can be controlled somewhat by adjusting the pH and the bisulfite concentration in the reaction. In a variation of this technique, DNA can be labeled by reacting the cytidine residues with biotin hydrazide in the presence of sodium bisulfite (*42*).

Psoralen derivatives of biotin (Figure 7) have also been used as labeling reagents for DNA (*43–45*). Psoralen has the ability to intercalate into double-stranded nucleic acids and, when irradiated with light (360 nm), form interstrand crosslinks. Probes suitable for labeling with psoralen–biotin derivatives must have an appropriate structure: a single-stranded region that will hybridize to the target and a double-stranded region that will carry the label. Usually, the psoralen moiety and biotin are separated by a spacer arm (Figure 7) to increase the availability of biotin for streptavidin binding. About one biotinylated psoralen is present in every 10 bp in the double-stranded region of the probe.

In recent years, it has been possible to synthesize short (<50 bases) single-stranded oligonucleotide probes by automation and to use these probes for hybridization or as primers for the polymerase chain reaction (PCR) or sequencing. These probes can be biotinylated via several different procedures. For end-labeling at the 3′-end, one can use the enzyme terminal deoxynucleotidyl transferase (TdT) and biotinylated nucleotides, e.g., biotin-11-dUTP (*46*). Alternatively, TdT can be used to introduce 4-thiouridine, which can then be reacted with haloacetamido derivatives of biotin, e.g., iodoacetyl-LC-biotin, to biotinylate the probes (*47*). RNA probes can also be labeled with biotin at their 3′-termini by first reacting the 2′,3′-*cis*-hydroxyls of the terminal ribose with periodate, to produce aldehyde groups that can then be reacted with biotin hydrazide as described above (Figure 3) (*48*). RNA probes have also been biotinylated at their 3′-termini by use of the enzyme T4 RNA ligase (*49*). End-labeling at the 5′ end

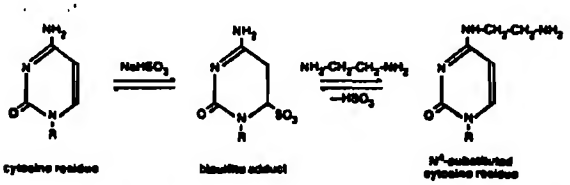

Fig. 6. Bisulfite-catalyzed transamination reaction with ethylenediamine
The N^4-substituted cytosine residue is biotinylated with NHS-LC-biotin as shown in Fig. 2

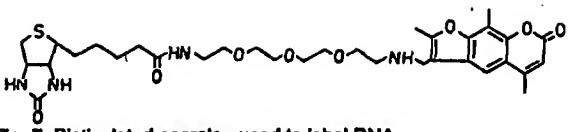

Fig. 7. Biotinylated psoralen used to label DNA
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of DNA has also been achieved with periodate oxidation (*50*): ribonucleotide monomers can be incorporated into short synthetic oligonucleotides by either the phosphotriester or the phosphoramidite routes and biotin-labeled with periodate oxidation and biotin hydrazide. Agrawal et al. (*50*) also synthesized another monomer (linker phosphoramidite), which could be incorporated at the 5'-end of a synthesized oligonucleotide. After deprotection, an $-NH_2$ group at the 5'-end of the oligonucleotide could be easily biotinylated with NHS-LC-biotin.

Many other methods can incorporate a free biotinylable amino group at the 5'-end of an oligonucleotide. For example, Smith et al. (*51*) used automated phosphoramidite chemistry to introduce deoxythimidine containing a linker arm with aliphatic amine.

By using the TdT tailing reaction, one can attach multiple biotins to the 3'-end of a synthetic polynucleotide. The products have greater sensitivity than probes singly biotinylated at their 5'-ends (*46*, *52*).

Newer biotinylation methods allow for biotin introduction internally into an oligonucleotide. For example, Jablonski et al. (*53*) and Haralambidis et al. (*54*) reported the synthesis of C-5-substituted deoxyuridines carrying a masked primary aliphatic amino group. These modified bases can be used as phosphoramidites in automated oligonucleotide synthesis (*52*); after deprotection, they possess a free amino group available for biotinylation. For better availability, this amino group should be attached to the base through a linker arm. With such procedures, both the location and the number of amino groups can be controlled. Similarly, C-8-substituted deoxyadenosine phosphoramidites have been reported (*55*). In yet more-straightforward procedures, biotinylated nucleoside phosphoramidites were synthesized (*56*, *57*) and used to produce by automated synthesis oligonucleotides that were multiply labeled at any site. More recently, nonnucleosidic phosphoramidite linker units carrying biotin have been synthesized and used to produce multiply biotinylated (e.g., eight biotins) short probes (*58*).

Cook et al. (*52*) examined in detail the achievable sensitivity in hybridization assays involving short 17-mer probes labeled with either one or up to three biotins at various sites internally or externally to the probe. They concluded that probes tailed with TdT gave the best overall results, whereas the poorest results were obtained with internally labeled probes. They also found that probes with internal biotins form less-stable hybrids than probes with external biotins. Others (*58*) also noticed that multiple biotinylation of short probes does not improve signal yield when streptavidin is the carrier of the label.

PCR is also a convenient method of producing relatively short labeled probes (*59*). During the PCR process, labeled nucleotides can be introduced into the final probe product. The enzyme Taq polymerase used in the PCR process incorporates biotinylated nucleotides, among others.

The degree of biotinylation of DNA, produced by any of the methods described above, can be assessed by at least two recently described procedures (*60*, *61*).

The Avidin–Biotin Interaction as a Detection System

The avidin–biotin interaction can be used for detecting a diverse number of targets with three different basic configurations (*62–64*):

(*a*) Avidin or streptavidin is labeled with a detectable molecule, e.g., an enzyme, fluorescent, chemiluminescent, or radioactive probe; a metal; or some other moiety. Biotin present in another reactant (antibody, nucleotide, Protein A, lectin, etc.) links the target molecule with the labeling system (Figure 8*A*). This detection format is used widely for immunoassays, DNA hybridization assays, immunohistochemistry, and flow-cytometry. Avidin and streptavidin conjugates carrying a variety of detectable molecules are commercially available (Table 2).

(*b*) Streptavidin is used unlabeled and serves to link the biotinylated binder with the biotinylated detection molecule (Figure 8*B*). This variation of the system takes advantage of the multiple biotin-binding sites in each avidin or streptavidin molecule. It is also used widely in immunoassay and DNA hybridization techniques, especially with probes that can be easily biotinylated (e.g., enzymes and proteinaceous fluorescent molecules).

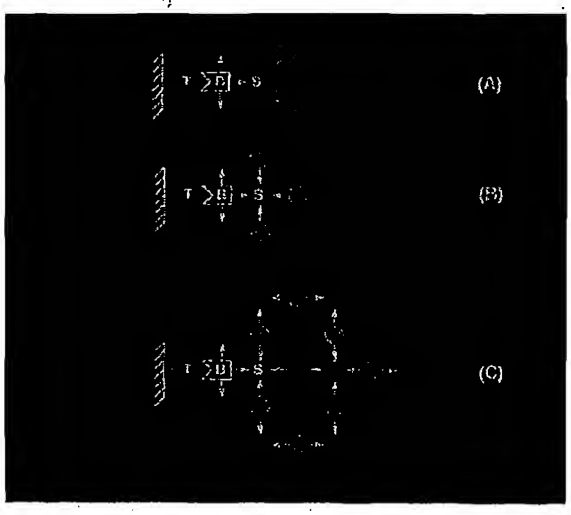

Fig. 8. Basic configurations of the biotin–streptavidin system
In all cases, the target molecule (T) is shown immobilized on a solid support. (*A*) The specific binder (B) of the target is biotinylated (→), and streptavidin (S) carries a label (O). (*B*) Same as (*A*) but streptavidin is used unlabeled to link the biotinylated binder and the biotinylated label. (*C*) Unlabeled streptavidin is mixed with biotinylated label to form a polymeric complex with some residual biotin-binding activity. This polymer binds to the biotinylated binder as in (*A*)

(*c*) The third method combines effectively the principles of (*a*) and (*b*) to yield a significantly more sensitive system (*3*). The basic idea is to mix, under controlled conditions, unlabeled streptavidin or avidin and a biotinylated detection reagent, e.g., an enzyme. Given the multiple biotin binding sites on avidin or streptavidin, one can generate a polymer having some free biotin-binding sites. This reagent is then used as in (*a*) but affords superior sensitivity. Kits offering the streptavidin or avidin and biotinylated enzymes at optimized concentrations, so that one can form the complex by simply mixing the reagents, are commercially available and are known as ABC (avidin–biotin complex) kits.

The Avidin–Biotin Interaction as an Affinity System

Immobilized avidin or streptavidin can be used to retrieve, purify, and characterize various moieties of interest (*2*). For example, a protein present in cells can be reacted with a specific biotinylated probe (antibody, receptor, lectin, hormone, etc.), which can then be retrieved by being placed in contact with a column or magnetic beads with immobilized avidin or streptavidin. The protein of interest can be released by disrupting the protein–biotinylated probe complex (*2, 26, 65, 66*). This approach can also be used with cleavable biotin analogs, facilitating easy release of the bound complex (*25, 37*). Biotinylated DNA can be retrieved by using similar procedures.

Avidin- or streptavidin-coated solid phases have been used effectively as separation agents in heterogeneous immunoassays (*67, 68*) and in DNA-hybridization assays (*69*). Two examples are given in Figure 9. Other applications include isolating and purifying DNA-binding proteins by use of streptavidin–DNA–biotin solid-phase (*70*), solid-phase DNA sequencing in combination with PCR (*71*), separation of cells tagged with biotinylated antibodies (*72*), and efficient fusion in hybridoma production (*73*) (see below).

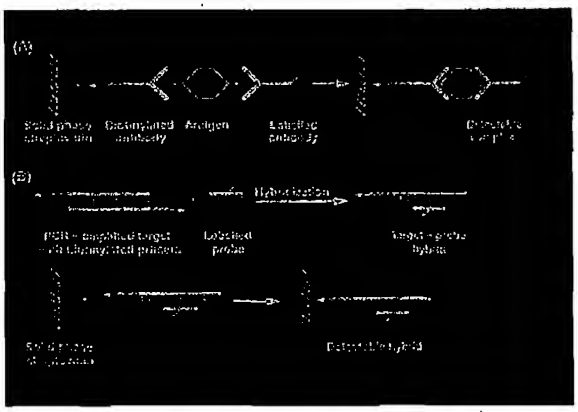

Fig. 9. Immobilized streptavidin used as a separation agent in immunoassays (*A*) and nucleic acid hybridization assays (*B*)
Washing removes unreacted detection antibody or labeled probe (not shown)

Selected Applications of the Biotin–(Strept)Avidin System

Immunological Assays

With the existence of biotinylation reagents and various avidin- or streptavidin-based detection systems (Tables 1 and 2), one would expect that many manual or automated avidin–biotin based immunoassays would be available commercially. This is not the case, however, and most of the available immunoassay kits or methods rely on direct labeling of the detection antibodies of interest. This is generally advantageous through avoiding the extra step of adding streptavidin label. On the other hand, the versatility of the avidin–biotin system makes it very attractive for research applications, as has been recently stressed in the literature (*74*), for the following reasons: For research applications, when appropriate antibodies are available, one of them can be immobilized on a solid support (e.g., polystyrene microtiter wells) and the other can be easily biotinylated as described before; streptavidin–enzyme conjugates, as well as many enzyme substrates, or other conjugates are available from many manufacturers; with such assay configurations (Figure 8*A*), excellent sensitivity can be achieved because of the amplification introduced by the avidin–biotin system. This amplification can be as high as 100-fold (10–20 active biotins per antibody and three to six enzymes per streptavidin). Competitive-type immunoassays can also be devised by using either the immobilized antigen approach and biotinylated antibody (*75, 76*) or the immobilized antibody approach and biotinylated antigens (*77*). In our hands, if good antibodies and calibration material are available, excellent assays can be set up in a few working days.

The most widely used enzyme conjugates for immunoassay are streptavidin–horseradish peroxidase (EC 1.11.1.7) and streptavidin–alkaline phosphatase (EC 3.1.3.1). With peroxidase, many substrates that yield colored products can be used (*78*). With alkaline phosphatase, the colorimetric substrate *p*-nitrophenyl phosphate, the fluorogenic substrate 4-methylumbelliferyl phosphate (*79*), or the chemiluminogenic substrate 3-(2′-spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy) phenyl-1,2-dioxetane, disodium salt (AMPPD) (*80, 81*), is used, the latter two having the better sensitivity. Recently, streptavidin conjugates have been used in time-resolved fluorometric applications (*82–89*). In one variation, Eu^{3+} is noncovalently attached to streptavidin by a strong Eu^{3+} chelator of the aminopolycarboxylic acid type (*82, 83*). After the immunological reaction is completed and excess biotinylated antibody is washed off, Eu^{3+}-labeled streptavidin is added (Figure 8*A*). The Eu^{3+} on the complex is then released in solution by lowering the pH and is re-complexed with ligands to form fluorescent complexes of long fluorescence lifetime. These complexes are quantified by using time-resolved fluorometry.

A different approach involves labeling streptavidin with the europium chelate of 4,7-bis(chlorosulfophenyl)-1,10-phenanthroline-2,9-dicarboxylic acid (*84–89*).

Streptavidin carrying a variable load of these chelates, ranging from 14 to 480 per molecule, has been synthesized and used to devise highly sensitive time-resolved fluorescence immunoassays.

Recently, Ishikawa et al. (*90*) used the avidin–biotin system to devise noncompetitive immunoassays for haptens. They biotinylated the hapten, which could then be bound by both its specific antibody and labeled streptavidin. Daunert et al. (*91*) devised a homogeneous assay for biotin, based on the observation that biotinylated glucose-6-phosphate dehydrogenase is inhibited if bound to avidin.

A very interesting variation of the avidin–biotin system in immunoassay has been developed by Bobrow et al. (*92*). In this assay, called catalyzed reporter deposition, horseradish peroxidase is used as label in a typical "two-site" immunoassay. However, the peroxidase, instead of producing a measurable signal, is used to catalyze the deposition of biotin-derivatives on the solid-phase, which is then detected by using streptavidin–enzyme conjugates. This approach amplifies manyfold the signal generated and shows promise for future applications that require very high sensitivity.

Many commercial sources apply the biotin–avidin technology not as a detection technique but as a high-affinity separation method of bound and free labeled reagent (Figure 9) (*67, 68*).

Nucleic Acid Hybridization Assays

The dominant labeling systems for various DNA-based assays are still radionuclides, i.e., ^{32}P and ^{35}S. These radionuclides offer superior sensitivity to that of the nonisotopic alternatives available today (*93*). However, ^{32}P and other radionuclides have some major disadvantages. Many laboratories, especially those interested in routine applications in the field of infectious disease, genetic disease, forensics, and cancer, are exploring alternative systems. Nonisotopic methodologies are now entering nucleic acid-based testing, with excellent prospects. In many applications (e.g., infectious disease), extreme sensitivity is not always needed; moreover, with the advent of PCR, the amplified product can be easily detected with nonisotopic methods. Some new nonisotopic assays claim to be as sensitive as ^{32}P assays (*94, 95*).

Because biotin can be easily and multiply incorporated into DNA without altering its ability to hybridize with its targets, it is the label of choice in many nonisotopic systems, with conjugated streptavidin being used for detection. Streptavidin–alkaline phosphatase conjugates have been widely used in combination with the colorimetric reagent NBT-BCIP (*94*), but more recently the chemiluminometric reagent AMPPD has been used for more-sensitive assays (*80, 95*). Streptavidin labeled with europium in combination with time-resolved fluorometry has also been used (*83*), or more recently, streptavidin labeled with europium chelates (*95*). From the information given before, one can easily deduce that any type of DNA assay (Southern, Northern, Western, dot–blot, etc.) can be performed by using the biotin–avidin interaction: biotinylated probes for Southern and Northern blotting and biotinylated antibodies for Western blotting.

Other Applications

The application of the system for DNA sequencing has recently been reported in different formats (*71, 96*). In one assay (*96*), based on the dideoxy chain termination sequencing method, biotinylated primers are used with direct blotting electrophoresis, which facilitates the transfer of the generated DNA fragments from the polyacrylamide gel to a nylon membrane (*97, 98*). The fragments, all containing the biotinylated primer, are then made visible by using streptavidin–alkaline phosphatase and the NBT-BCIP substrate.

In flow cytometry, biotinylated antibodies are used to react with specific membrane targets; the cells are made fluorescent by further reaction with streptavidin–fluorochrome conjugates. In receptor-hormone studies, the hormone can be biotinylated, provided biotinylation does not alter its ability to bind to the receptor (*99*). In electron microscopy, ultrastructure can be localized by using specific antibodies and avidin–gold or avidin–ferritin conjugates or unlabeled streptavidin and biotinylated ferritin (*31, 100*).

More recently, some new reagents have been described for use with the avidin–biotin system. Anti-biotin antibodies can be used when the high affinity of the avidin–biotin interaction is not desirable (*35*). In this case, the biotin moiety is used as a hapten in a manner similar to other "hapten–anti-hapten" systems, e.g., the digoxigenin system (*101*). Antibodies against avidin and streptavidin have also been prepared and are available conjugated with enzymes or biotinylated. Biotinylated anti-avidin and anti-streptavidin are interesting reagents because they can react with avidin or streptavidin from two different sites. They can thus be used as amplifiers, as described elsewhere (*102–105*).

Another novel application of the biotin–avidin system has been the facilitated cell fusion for hybridoma production (*73*). In this method, biotin is attached to the membrane of myeloma cell lines used for hybridoma production. An antigen–avidin conjugate is mixed with the spleen cells suspension that is producing the antigen-specific antibodies. The spleen cells producing antibodies express the antibodies on their surfaces and are selectively labeled with avidin. Mixing the biotinylated myeloma cells and the avidin-labeled antibody-producing spleen cells yields complexes of the form myeloma cell–biotin–avidin–antigen–spleen cell, which will fuse efficiently by electrofusion. The close proximity of myeloma cells with antibody-producing spleen cells (but not non-antibody-producing spleen cells) facilitates greater yields during electrofusion. Alternatively, the myeloma cells can be avidinylated and the antigen of interest can be biotinylated and attached to the antibody-producing spleen cells.

Other interesting applications of the system for targeted imaging and drug delivery in combination with specific antibodies have been reported (*10, 106*).

In conclusion, the extreme versatility of the avidin–biotin system and the commercial availability of many auxiliary reagents will undoubtedly contribute to the more widespread use of this system in diverse areas of biotechnology.

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EXHIBIT D

Generation of Periodic Enzyme Patterns by Soft Lithography and Activity Imaging by Scanning Electrochemical Microscopy

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Micropatterns of the enzymes glucose oxidase (GOx) and horseradish peroxidase (HRP) have been formed on polycrystalline gold and glass surfaces using different soft-lithographic approaches. The patterns have been analyzed by noncontact scanning force microscopy. The activities of the immobilized patterns were probed with local resolution by scanning electrochemical microscopy. The following approaches have been tested: (i) microcontact printing of octadecanethiol on gold, followed by chemisorption of cystaminium chloride and incubation of a mixture of HRP and glutaraldehyde to form a patterned cross-linked and grafted enzyme gel; (ii) microcontact printing of octadecanethiol on gold followed by chemisorption of HRP into which an thiol group had been introduced by modification with *S*-acetylthioglycolic acid-*N*-succinimidyl ester; (iii) application of a mixture of GOx with *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) to a stamp and contact with an aminated glass surface; (iv) delivery of a mixture of GOx with EDAC through microscopic open channels in a block of poly(dimethylsiloxane) pressed against an aminated glass surface. Satisfying contrast in enzymatic activity and high local enzyme activities were achieved by the modification of the gold surfaces. A general advantage of this monolayer system is the speed with which the binding of thiols to gold occurs. Therefore, the patterning step can be carried out with short contact times and the enzyme incubation can be made under controlled conditions in a moisture chamber. Approach ii offers the special advantage of reducing the number of process steps to two (stamping and application of thiolated enzyme), making it suitable for building up more complex structures formed by a sequence of structuring steps.

1. Introduction

Micropatterns of biomolecules receive increasing interest due to demands for parallel testing systems and continuing development of miniaturized biosensors, biosensor arrays, and chip-based testing systems. While DNA-based testing systems have already reached the commercial market, equally advanced testing systems using proteins as functional units are more difficult to construct because the proteins typically are much less stable and may denature upon contact with the surface onto which they are to be immobilized. Approaches have been pursued along several strategies. From the coupling chemistry entrapment in polymers and gels, cross-linking or attachment to the terminal group of a self-assembled monolayer (SAM) have been tested. From the structuring technology, site-directed immobilization is performed by attachment to microstructured substrates,[1−3] generation of extended periodic patterns using lithographic approaches,[4,5] microcontact printing and subsequent modification,[6,7] local electrochemical activation using an electrode array as a patterned reagent generator,[8] or site-selective supply of biomolecules by microfluidic networks.[9,10] Local modification by interaction with scanning probes includes a large variety of interactions, for example, local electrochemical desorption[11] of alkanethiolate SAMs and further chemical modification;[12,13] local electrochemical deposition of gold and functionalization by thiolate chemisorption,[14] by removal of adsorbates by scanning probe tips,[15−17] or by writing alkanethiolate layers using a scanning force microscope tip and cantilever as a dip-pen;[18] local electropolymerization of conducting polymers with entrapment of an enzyme;[19,20] or delivery of picoliter droplets with enzyme solution to an activated specimen

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surface by piezoelectric fluid pumps (ink-jet principle).[21,22] Recently, MacBeath and Schreiber created arrays of functional proteins by transferring drops of an enzyme solution onto an activated glass surface by an arrayer robot.[23] The proteins were functional with a spot size of about 150−200 μm in diameter which was limited by the surface area wetted by a nanoliter droplet transferred by the arrayer robot. The site-directed assembling of surface-functionalized, magnetic microbeads was introduced by Wijayawardhana et al. and used in different scenarios.[24−26] The local modification enables the generation of microregions with different functionalities onto one support surface.

Periodic micropatterns are desirable for a variety of applications. Such situations include microcompartmentalized surfaces which can unite different interdependent functionalities onto one support such as enzyme-modified regions and bare electrode surfaces for sensors with fast response times[27] or immobilization of several enzymes with different requirements with respect to the local environment. For generation of such patterns, lithographic processes possess several advantages. Usually macroscopic regions can be modified by a micropattern in one process step; patterns can be easily reproduced on several specimens. While usually restrictions exist in the adaptability of the pattern layout, the modification can be very fast once masks or stamps are available. Microcontact printing (MCP) has gained popularity for micropatterning of SAMs.[28] A fraction of the surface defined by the contact of the specimen with a polymer stamp (typically from poly(dimethylsiloxane), PDMS) is wetted with an *n*-alkanethiol. Rinsing the surface with an ω-functionalized thiol/disulfide forms a bifunctional pattern to which proteins can be coupled. Such a procedure is fully compatible with the handling of sensitive enzymes and hence has been used for immobilizing proteins for various applications.[6,7] Although not restricted to alkanethiols on gold, this system received particular attention as a starting point for building biochemically active surfaces. It allows the binding of functional units in a defined distance to the electrode which may be used to facilitate direct electron transfer[29,30] or arrangement of supramolecular, catalytically active entities on surfaces.[31] The approach has been expanded: The term "soft-lithographic techniques" comprises a number of techniques using a microscopic polymer stamp either to transfer reagents to a surface or to form defined reaction moulds or transport channels on the surface to be modified.[28] Direct stamping of proteins has been reported.[32−34]

This contribution compares the potential of four soft-lithographic strategies to generate periodic patterns of *active* enzymes: (i) A bifunctionalized surface created by microcontact printing of a methyl-terminated alkanethiol and rinsing with an amino-functionalized disulfide is converted to an micropatterned enzyme layer by anchoring horseradish peroxidase (HRP) and glutaraldehyde to the amino functionalities. (ii) After stamping of an alkanethiol, free gold areas were covered by chemisorption of HRP into which SH-groups were introduced by reaction with *S*-acetylthioglycolic acid *N*-hydroxy succinimidyl ester (SATA). (iii) By stamping a mixture of glucose oxidase (GOx) and carbodiimide onto an aminated glass surface, a cross-linked enzyme gel was formed. (iv) This was compared to the micromolding in capillaries (MIMIC) by which a GOx−carbodiimide mixture was incubated in channels of a PDMS stamp which was pressed onto aminated glass.

The local enzyme *activity* of the patterns is analyzed using the generation−collection (GC) mode of the scanning electrochemical microscope (SECM). In this working mode, an amperometric ultramicroelectrode (UME) is used to detect a metabolite of the enzyme. The faradic current at the UME is a measure of the local enzyme activity underneath the UME. The application of the SECM for imaging local enzyme activity was reviewed recently.[35−37] The choice of enzymes was governed by the possibility of imaging the activity of the immobilized enzymes by scanning electrochemical microscopy. Although potentially a large number of enzymes can be investigated by this technique, it has been previously demonstrated just for a few. Extension of this palette has been demonstrated by others and us.[38,39] It requires finding suitable mediators that are cofactors of the enzyme but can be detected at an UME with high stability. Furthermore, a minimum specific activity is required for successful SECM imaging. In this paper, emphasis is put on geometric patterns of enzymes that could be potentially further modified. Enzymes have been selected that have a high specific activity and for which existing optimized SECM imaging protocols do exist. In the further course of the project, we aim to achieve a local modification of the periodic patterns formed by the soft-lithographic approach and to follow the local activity by SECM imaging. To demonstrate this approach with SECM imaging of the local enzyme activity, rather large structures with scales of about 100 μm were selected for the soft-lithographic techniques. This should allow convenient positioning of the UME above individual structures for local surface modification and activity imaging. Downscaling the patterning procedures by a factor of more than 10 has already been demonstrated and should be no problem with the approaches presented here. However, local activity imaging in the GC mode of the SECM is unlikely to yield clearly resolved features if such small features are formed with inert regions of about the same dimension between the enzyme-modified areas.

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2. Experimental Section

2.1. Chemicals. Glass slides were standard microscopic slides from Roth (Karlsruhe, Germany). The following chemicals were used as received: chromium (99.999%, Haeraeus, Karlsruhe, Germany), gold (99.99%, Degussa, Hanau, Germany), 3-aminopropyltriethoxysilane (purum, Fluka, Buchs, Switzerland), toluene (puriss. abs., Fluka), octadecanethiol (pract., Fluka), dodecanethiol (purum, Fluka), glutaraldehyde (50% aqueous solution, Fluka), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC, purum, Fluka), sorbitan monolaureate (Tween20, Serva, Heidelberg, Germany), D-(+)-glucose (MicroSelect, Fluka), cystaminium chloride (98%, Aldrich, Deisenhofen, Germany), glucose oxidase (type X-S, *Aspergillus niger*, Sigma), horseradish peroxidase (type VI-A, Sigma), hydroxymethylferrocene (ABCR, Karlsruhe, Germany), diethyl ether (absolute, analytical, Merck, Darmstadt, Germany), ethanol (98%, analytical, Merck), toluene (purum, Merck), Na_2HPO_4 (analytical, Fluka), $NaH_2PO_4 \cdot H_2O$ (analytical, Merck), H_2SO_4 (96%, analytical, Fluka), HNO_3 (analytical, Fluka), NaOH (analytical, Merck), KCl (analytical, Merck), $K_4[Fe(CN)_6] \cdot 3H_2O$ (analytical, Merck), H_2O_2 (30% aqueous solution, analytical, Merck), poly(dimethylsiloxane) (Sylgard 184, Dow Corning, LaHulpe, Belgium). SATA was prepared as described in the literature[40,41] and characterized by ^{13}C NMR, ^1H NMR, MS, and elemental analysis. Aqueous solutions were prepared using deionized water obtained from a water purification system (Christ, Stuttgart, Germany).

2.2. Sample Preparation. Au surfaces were prepared by evaporation of 30 nm of Cr and 1500 nm of Au onto glass slides using a Tectra minicoater evaporation chamber (Tectra, GmbH, Frankfurt, Germany). To obtain amino-functionalized glass surfaces, slides were immersed sequentially for 12 h in a mixture of 1 volume of 20% (mass/mass) NaOH and 1 volume of 96% (v/v) ethanol and for 4 h in a mixture of 1 volume of concentrated H_2SO_4 and 1 volume of concentrated HNO_3. After washing the slides for 20 min in water in an ultrasonic bath (30 W) and drying at 105 °C for at least 2 h, the slides were immersed in a solution of 0.5 g of $(C_2H_5O)_3Si(CH_2)_3NH_2$ in 50 mL of toluene for 12 h and ultrasonicated sequentially in toluene (5 min), ethanol (5 min), and water (20 min). Functionalized slides were stored dry at 60−80 °C.

2.3. Surface Patterning. For the microcontact printing of thiols, the stamp was inked with a 1 mM solution of thiol in diethyl ether and dried for 2 min. After contact with the surface for 1 min, the stamp was removed and the surface was rinsed with diethyl ether (five times), methanol (twice), and water (twice). Amino functionalization of the bare gold surface was performed by exposure of the sample to a 1% (mass/mass) aqueous solution of cystaminium chloride (30 min) and subsequent rinsing with water (five times). Coupling of HRP to the amino-terminated surface was achieved by incubating a mixture of 100 μL of 5% (mass/mass) aqueous glutaraldehyde solution and 1 mg of HRP in 100 μL of phosphate buffer (pH 7) onto the surface within a moisture chamber for 2 h. Afterward the sample was rinsed with Tween20 solution (0.2% (v/v) in phosphate buffer, pH 7) twice and five times with phosphate buffer (pH 7). For direct surface modification of bare gold areas with enzyme, the SATA−HRP conjugate was prepared as described in the literature.[40,41] The conjugate solution was then delivered onto the alkanethiolate/bare gold surface, and the sample was incubated in a moisture chamber for 30 min. Subsequently the sample was rinsed with Tween20 solution (same as above, once) and five times with phosphate buffer (pH 7).

To stamp GOx directly onto amino-functionalized glass, the stamp was wetted with a solution of 4 mg of GOx and 12.5 mg of EDAC in 0.5 mL of phosphate buffer (pH 7), dried for 5 min, and put onto the sample for 30 min. After removal of the stamp, the sample was rinsed with Tween20 solution (same as above, once) and five times with phosphate buffer (pH 7). For micromoulding in capillaries, the same solutions were incubated in the mould for 2 h and the same rinsing protocol was used.

2.4. Surface Characterization. Scanning force microscope (SFM) images were obtained using a Rasterscope 4000 (DME, Herlev, Denmark) in the noncontact mode. A home-built SECM instrument has been used. It consists of a stepper motor system (Scientific Precision Instruments, Oppenheim, Germany) with reduced backlash due to a special polymer coating around the moving parts and a specifically designed monopotentiostat (M. Schramm, Heinrich Heine University of Düsseldorf, Düsseldorf, Germany). The monopotentiostat was found to give superior performance for imaging of enzymes when currents below 1 nA are found and no second working electrode is needed. The system is controlled by a PC and a DAS 1602-16 A/D card (Plug-In-Electronic, Eichenau, Germany). Glass-enclosed platinum wires of 10 and 25 μm diameter were used as the UME. The ratio, RG, between the radii of the glass and the active electrode area, r_T, ranged between 9 and 15 and was achieved by grinding and polishing the electrodes in a home-built polishing station built according to the recommendation of Kranz et al.[42] A Pt wire as auxiliary electrode and a saturated calomel electrode (SCE) completed the electrochemical three-electrode setup for SECM imaging experiments. All electrochemical potentials are given with respect to the SCE. Feedback images were obtained in 5 mM $[Fe(CN)_6]^{4-}$ + 0.1 M KCl solution at a UME potential of E_T = 600 mV or in 2 mM hydroxymethylferrocene + phosphate buffer + 0.1 M KCl at E_T = 400 mV while the sample was at open circuit potential (OCP). For imaging of HRP activity in GC mode at E_T = 0 mV, the electrolyte solution contained 0.5 mM H_2O_2 + 5 mM hydroxymethylferrocene + 0.1 M KCl. The imaging of GOx activity in GC mode was performed in 50 mM glucose + 0.1 M KCl + phosphate buffer (pH 7, air saturated) at E_T = 750 mV. Images and profiles of SFM and SECM data were generated with the in-house-developed software MIRA.[43] A linear background was subtracted from the images in Figures 2 and 4. Figures 1, 3, and 5 are shown as recorded.

3. Results and Discussion

3.1. Characterization of Stamped Alkanethiolate Monolayers on Gold Using the SECM. The fabrication of a patterned thiolate monolayer by MCP and subsequent coupling of enzymes to the remaining bare gold area can be regarded as a two-step approach to generate periodic enzyme patterns. This implies potential for optimization of both the MCP and the covalent coupling. Since the function of the patterned initial monolayer is to prevent the enzyme or spacer from reaching the reactive surface and binding there, the contrast and defect density of the stamped SAM are crucial to ensure high pattern quality. Experiments have been performed with long-chain alkanethiols and ω-mercaptohexadecanoic acid. For the experiments, patterns were stamped with a contact area of 100 × 100 μm^2 squares with 50 μm distance between (Figure 1a). The local quality of the patterned monolayer can be characterized using the SECM feedback mode (Figure 1b) because a dense thiolate SAM prevents the contact of solutes with the gold surface. Therefore, heterogeneous electron-transfer reactions of a dissolved redox mediator with the gold surface are inhibited. When the UME is located above stamped monolayers of high quality, the UME current i_T should behave similar to that over an inert insulating surface by falling below its value in the bulk phase ($i_{T,\infty} = 4\,nFDc^*r_T$, with symbols assuming the following meanings: n, number of exchanged electrons; F, Faraday constant; D, diffusion coefficient; c^*, mediator bulk concentration; r_T, UME radius). The hindered diffusion above inert insulating samples is characterized by $i_T/i_{T,\infty} < 1$, frequently called "negative feedback". An SECM image of a stamped ω-mercaptohexadecanoic acid layer meets this expectation (Figure 1c). High currents are observed above the grid lines of bare gold where no contact

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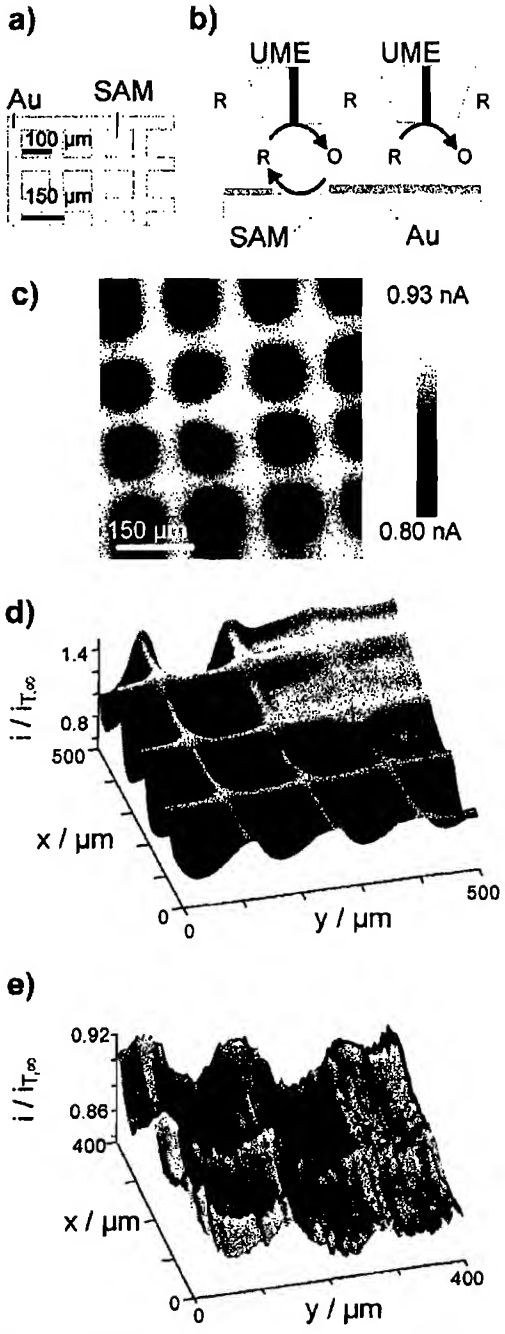

Figure 1. SECM feedback imaging of a stamped methyl-terminated SAM. (a) The contact areas were squares of 100 × 100 μm^2 with a 50 μm spacing between; contact time, 1 min. (b) Schematic of the SECM experiment; R, reduced mediator (Fe^{2+} complex); O, oxidized mediator (Fe^{3+} complex); r_T = 12.5 μm, E_T = 600, sample is at OCP. (c) Pattern obtained from stamping ω-mercaptohexadecanoic acid ($i_{T,\infty}$ = 0.881 nA); solution, 2 mM hydroxymethylferrocene + phosphate buffer (pH 7) + 0.1 M KCl. (d) Pattern obtained from stamping octadecanethiol ($i_{T,\infty}$ = 11.5 nA) illustrating the ability to detect defects within the stamped monolayer; solution, 5 mM $[Fe(CN)_6]^{4-}$ + 0.1 M KCl. (e) Pattern obtained from stamping dodecanethiol ($i_{T,\infty}$ = 0.557 nA); solution, 1 mM $[Fe(CN)_6]^{4-}$ + 0.1 M KCl.

beween stamp and gold occurred. The spacing between the lines reproduces the periodicity of the stamp. As demonstrated in Figure 1d, the current above the stamped area is very sensitive to imperfections in the monolayer, in particular if small UME–sample distances are used for imaging. The image shows a good contrast between alkanethiolate-covered regions and bare gold areas with signal differences of up to 9.7 nA. The current above the bare gold areas amounts to 16.7 nA and is well above $i_{T,\infty}$ = 11.5 nA. The high current in the area $x > 300$ μm, $y > 200$ μm reveals an irregularity in the patterned SAM, probably caused by a cotton fiber or dust particle which rested between stamp and surface during stamping and prevented contact between the PDMS and the Au surface locally. Therefore, the corresponding area was not modified to the same extent like the other contact areas. However, the current is slightly reduced compared to the values above the grid of the bare gold. Previously we used galvanic copper deposition, formation of black copper sulfide by immersion into Na_2S solution at OCP, and optical microscopy to test the quality of patterned SAMs.[13] This procedure is fast and requires a minimum of instrumentation. However, the sample is altered both by the deposition of another material and possibly by application of an electrochemical potential to the monolayer-covered gold surface when driving the copper deposition. Methods based on etching the unprotected gold layer share similar limitations.[44] Characterizing the defects in the SAM by the inhibition of the electron-transfer reaction of a dissolved quasi-reversible redox couple requires imposing electrochemical potential to the SAM-covered gold that might alter the SAM itself.[46–48] In this respect, SECM feedback imaging is a very good alternative for testing the quality of the SAM. It is sensitive to defects in the same way as cyclic voltammograms but does not require the application of external potentials to the SAM-covered gold electrode. A pronounced negative feedback is only observed above very well prepared monolayers. The main disadvantage of the SECM imaging is the comparatively low speed at which an image frame with large scanning ranges can be recorded.

Obvious optimization parameters for MCP experiments are the choice of the thiol and the contact time between stamp and surface. As described in the literature,[49,50] long-chain (C_{16}–C_{20}) thiols form denser monlayers than short-chain (C_8–C_{11}) thiols and are less likely to be transported via the gas phase during stamping.[51] On the other hand, thiols of medium chain length (C_{12}) are most suitable for microstructuring by electrochemical desorption.[11,13] Since one of the goals was to obtain patterns suitable for further modification by electrochemical desorption, attempts were made with shorter alkanethiols as well. For these thiols, no compromise has been found so far between the time needed for the formation of a dense thiolate film with inhibiting properties and the contribution of gas-phase transport of the thiol leading to an undesired coverage of

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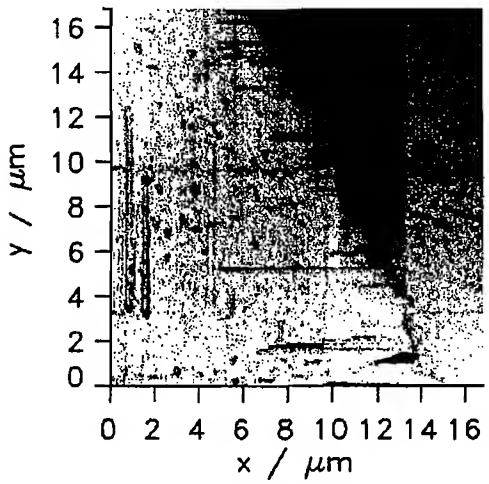

Figure 2. Noncontact mode SFM image of the edge of a glutaraldehyde-cross-linked HRP layer grafted to an amino-terminated monolayer. The horizontal and vertical lines are imaging artifacts.

the noncontact areas.[51] A typical example of a stamped dodecanethiolate layer is given in Figure 1d. The SECM image has a poor contrast with a signal difference of only 0.03 nA (0.49−0.52 nA). Furthermore, the current above the entire image frame is lower than $I_{T,\infty} = 0.557$ nA. The gas-phase transport of the thiol during the stamping and the poor film density of the dodecanethiolate monolayer formed during the short contact time render this thiol unsuitable for further experiments. We found carboxylic acid terminated SAMs (Figure 1c) to be quite efficient in inhibiting electrochemical reactions while avoiding gas-phase transport during stamping. In contrast to long-chain alkanethiolate SAMs, they can be modified by local electrochemical reactions similar to that shown in ref 13.

3.2. Amination of the Bare Au Surface and Coupling of HRP with a Cross-Linker. After octadecanethiol was stamped to a gold surface, an enzyme-patterned surface was created by chemisorbing cystaminium chloride $[H_3N-(CH_2)_2-SS-(CH_2)_2-NH_3]Cl_2$ and coupling the enzyme covalently to the aminated regions of the surface. Several protocols have been explored for this step. They include the direct coupling of periodate-oxidized GOx containing aldehyde groups in the carbohydrate part of the enzyme to the amino groups of the monolayer.[12] The same enzyme has been coupled to amino groups on the surface with the help of carbodiimide as an activator.[52] Another approach consists of binding glutaraldehyde to the aminated surface in order to create an aldehyde-terminated surface which can be used to couple to amino groups of the enzyme in a second incubation step.[53] Here we explore the possibility of incubating a mixture of glutaraldehyde and the enzyme HRP on the aminated surface.[38] Glutaraldehyde as a bifunctional reagent will couple to the terminal amino groups of the enzyme and to the aminated surface. Because the enzyme contains more than one accessible amino group, enzyme molecules will also be cross-linked. As a consequence, a thin gel-like polymer is formed that can be imaged by noncontact SFM measurements (Figure 2). The edge between the enzyme gel and the methyl-terminated surface is running from (x/μm, y/μm) = (8, 16) to (14, 0) of Figure 2. The stripes parallel to the x and y axes are imaging artifacts formed when the sharp tip is abrading the gel and gel fragments temporarily adhere to the tip. The image was recorded with the high-frequency axis parallel to the x axis. The area had been analyzed with the high-frequency scan axis rotated by 90° before. Therefore, stripes with a 90° angle to each other can be found in the image frame. The thickness of the gel layer as taken from a profile amounts to 60 nm. This is in good agreement with results of Kratzmüller et al. who formed and characterized polypeptide films of 32 nm thickness using a similar procedure.[54]

The enzymatic activity of microscopic gel structures with a layout shown in Figure 3a can be probed by the GC mode of the SECM. The bulk solution contains H_2O_2 and hydroxymethyl ferrocene, and the UME is held at E_T = 0 V in order to detect hydroxymethyl ferrocinium ions formed at the HRP gel by reduction without interference from H_2O_2 or dissolved O_2 (Figure 3b). In the presence of H_2O_2 and hydroxymethyl ferrocene, high reduction currents are found along the grid lines (Figure 3c). This corresponds to the expectation from the monolayer layout with a periodicity of 150 μm and a square grid of HRP-modified lines. Because of the large imaging times for large image frames and problems in avoiding a tilt, small sections were investigated in more detail (Figure 3d). The upper image of Figure 3d shows the lines of immobilized HRP by high reduction currents along the lines x = 100 mm, x = 250 μm, and y = 125 μm. The squares, which were initially modified by a methyl-terminated alkanethiol, are not modified by the enzyme and give rise to little currents at the UME. A control experiment was conducted in order to verify that the signal is due to the enzymatic reaction and not due to SECM feedback with the underlying gold showing less inhibition than over the methyl-terminated SAM with an impurity of the solution as a mediator (e.g., traces of air-oxidized hydroxymethyl ferrocene). The result is shown in Figure 3d, lower image. The current decreased almost to zero. The remaining very weak current enhancements over the gridlines are due to an incomplete solution exchange in our cell with some compartments for reference and counter electrodes that form dead volumes during the solution exchange. During initial experiments, some problems were encountered to avoid mechanical shifts of sample versus tip location during the solution exchange. The small remaining features in Figure 3d, lower image, serve as proof that the same sample region was imaged before and after the solution exchange. Therefore, no attempts were made to eliminate them completely.

The resolution of the GC mode of the SECM (Figure 3d) is much lower than the resolution of the SECM feedback imaging of alkanethiolate monolayers (Figure 1d) with a comparable UME size. These are well-documented features of the two imaging modes.[36,37,55] The GC mode, however, provides a much better sensitivity and allows imaging of enzymes immobilized on metal surfaces.[12] During feedback imaging of such systems, there would be a superposition of the mediator recycling by the electrochemical reaction at the metal surface and the mediator recycling by the enzymatic reaction, with the electrochemical reaction contributing by far the bigger part to the signal.[20] For these two reasons, the GC mode had to be selected for imaging of the immobilized enzyme activity despite its inherent lower resolution compared to the

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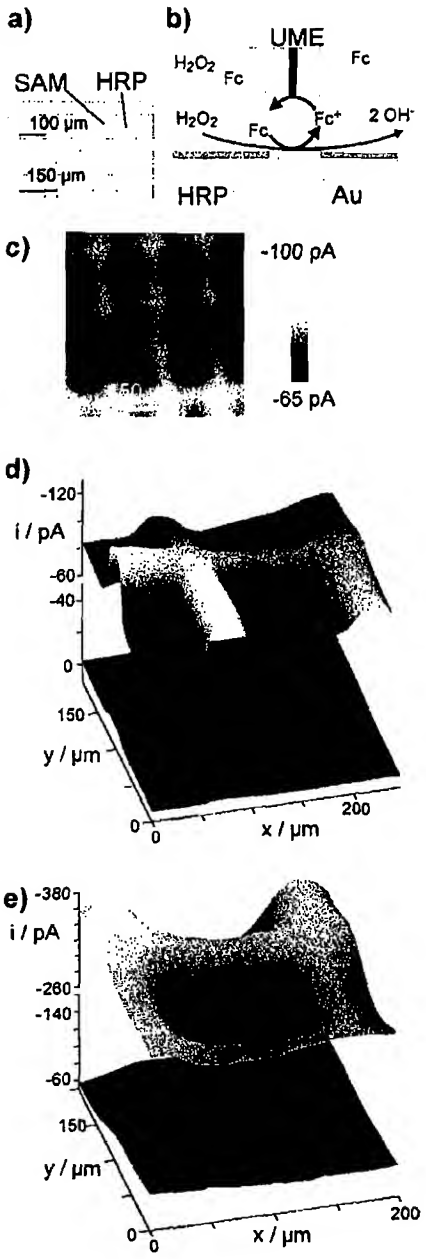

Figure 3. SECM imaging of localized HRP activity on patterned SAMs on gold. (a) Layout; HOOC–$C_{15}H_{30}$–SH (c) or $C_{18}H_{37}SH$ (d,e) was applied at contact areas that were squares of 100 × 100 μm^2, and HRP was bound to the 50 μm wide lines between them. (b) Schematic of the SECM experiment; Fc, hydroxymethyl ferrocene; Fc^+, hydroxymethyl ferrocinium ion. (c) SECM image of HRP activity; for immobilization, after a rinse of the surface with cystaminium chloride, HRP was immobilized by incubation with glutaraldehyde, in a solution of 0.5 mM H_2O_2 + 5 mM hydroxymethylferrocene + 0.1 M KCl. (d) SECM image of HRP activity; top, same as (c); bottom, solution was exchanged with 5 mM hydroxymethyl ferrocene + 0.1 M KCl without H_2O_2. (e) SECM image of HRP activity. HRP was first modified with SATA and activated by hydroxylamine to introduce a thiol group into the enzyme. The modified enzyme binds directly to the bare gold regions; top, 0.5 mM H_2O_2 + 5 mM hydroxymethyl ferrocene + 0.1 M KCl; bottom, solution was exchanged with 5 mM hydroxymethyl ferrocene + 0.1 M KCl without H_2O_2; for all images, r_T = 12.5 μm, E_T = 0 mV.

feedback mode. The SFM image in Figure 2 illustrates that the edge definition is much better than the resolution of the GC images in Figure 3. This applies equally to the GC images in Figures 4 and 5.

The advantage of the coupling procedure consists of the fact that the coupling of the enzyme is done in three steps, stamping of the methyl-terminated monolayer, chemisorption of cystaminium dihydrochloride, and coupling/cross-linking of the enzyme with glutaraldehyde, whereas four steps would be required if glutaraldehyde is reacted first with the amino groups at the surface and then in a separate step with the amino groups of the enzyme avoiding cross-linking. Incubation of an enzyme–glutaraldehyde mixture leads to the formation of multilayer enzyme gels which can accommodate high enzyme amounts. However, cross-linking is generally believed to decrease the specific enzyme activity. On the other hand, this is a feature common to almost all the covalent coupling procedures in question for monolayer modification.

3.3. Modification of Bare Au Surface Areas with SATA-Modified HRP. For creating more complicated patterns, there is a clear need to further reduce the number of incubation steps for the immobilization of biological functions on patterned monolayers. This can be reached by a homogeneous reaction of the enzyme with SATA (Scheme 1). A free thiol group is formed after deacetylation of the protein–SATA conjugate by hydroxylamine (Scheme 2). As a result, the protein contains an accessible thiol group and can therefore directly be chemisorbed on the gold surface. Since this reaction does not involve amino groups on the surface to be modified, coupling to existing enzyme films is not to be expected. Furthermore, there is no cross-linking of the enzyme being immobilized this way which prevents loss of activity due to cross-linking. However, it is not a trivial question whether the enzyme will retain its activity because loss of catalytic activity frequently occurs when enzymes adsorb onto metal surfaces and this induces changes in the tertiary protein structure to maximize the interaction with the metal surface.[56] The corresponding experiments have been made by introduction of a thiol group into HRP and chemisorption of the modified enzyme to gold areas unmodified after initial MCP of octadecanethiol on gold (Figure 3a). SECM imaging of the local activity allows verification that chemisorbed HRP is still catalytically active (Figure 3e). The stamp layout and imaging conditions were the same as used for Figure 3d. Enhanced reduction currents are seen in Figure 3e, upper image, at x = 25 μm and x = 175 μm as well as y = 0 μm and y = 150 μm corresponding to the layout of the stamp. The control experiment after rinsing out most of the H_2O_2 proves that the signal is caused by the catalytic conversion of the enzyme.

The chemisorption of SATA-modified enzymes represents a new approach to form patterned enzyme layers with soft-lithographic techniques. It has special advantages if the number of process steps needs to be minimized. A further advantage is that once the SATA–enzyme conjugate is formed in a homogeneous reaction, free amino groups are not required or used when binding the enzyme to the surface. When aiming at multienzyme structures, this is an important feature because otherwise the second enzymes would bind to terminal amino groups at the SAM but also to amino groups of already attached protein layers.

3.4. Direct Stamping of GOx and an Activating Agent onto Aminated Glass. The possibility of exchanging the gold films as supports with glass would have

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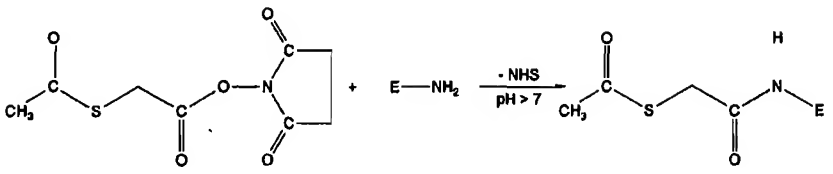
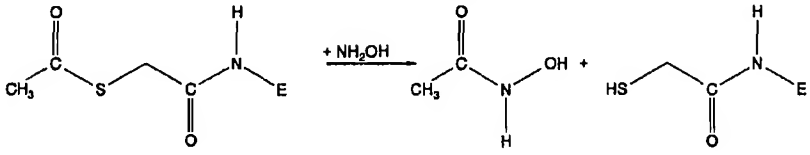
Scheme 1



(1)

Scheme 2



(2)

several advantages besides the lower price of the material. Glass is transparent and would allow easy optical inspections of samples and convenient access to observe phenomena like chemiluminescence.[58,59] SECM imaging would benefit from the possibility of applying the enzyme-mediated feedback effect. This imaging mode can provide much better lateral resolution. It is, however, not suitable for enzymes immobilized on conducting supports because the contributions of electrochemical and enzyme-mediated mediator recycling interfere.[12,36,37] To create patterned enzyme layers on a glass support, the possibility of using soft-lithographic techniques was explored. The main difficulty is that the formation of monolayers requires more time than in the alkanethiol–gold system. Long contact times in return create other problems such as loss of activity, pattern degradation by gas-phase transport or surface diffusion. Finally, the required small amount of water[60] is difficult to control in a soft-lithographic experiment. Therefore, a batch of glass was aminated over the whole surface. A PDMS stamp was then wetted with a mixture of the enzyme GOx and carbodiimide as an activator. The solvent water was allowed to dry. The stamp with the almost-dry enzyme was brought into contact with the aminated glass and left there for 30 min. During this time, coupling of the GOx to the aminated glass occurred on the square contact areas between stamp and glass (Figure 4a). Cross-linking between the enzyme molecules has to be expected as well. The activity of the immobilized enzyme can be investigated in the GC mode of the SECM (Figure 4b). The air-saturated solution contains glucose, and the UME detects the local formation of H_2O_2 by the GOx. In the presence of glucose in the solution, higher oxidation currents are measured over the squares where the stamp contacted the glass (Figure 4c, upper image). The periodicity of the pattern in the image corresponds to the layout of the PDMS stamp. In the absence of glucose in the solution, the currents are reduced to almost zero, proving that the signals in the upper image originate from the enzymatic reaction. The remaining weak features result from incomplete solution exchange and confirm the identity of the imaged regions before and after solution exchange (see the discussion of Figure 3d above). A profile across three GOx-modified regions (Figure 4d) shows that the difference between currents is very small (a few pA). Even without subtracting a rising background from Figure 4c, upper image, the overall current level is small compared to that of our own previous experiments, where GOx was attached to an amino-functionalized gold surface.[12,57] The current rises in the y (low-frequency) direction in which the image was recorded. This is most likely due to a slow accumulation of H_2O_2 during recording of this large image frame. Sample tilt might add another contribution to this effect. The H_2O_2 accumulation in GC imaging of GOx has been proven earlier.[12] The overall low enzymatic activity is most likely due to the harsh conditions during the contact printing where the enzyme solution was left to dry on the PDMS stamp and left in contact for 30 min on the aminated glass. This time is still short compared to the recommendations of many protocols for coupling proteins to amino groups by carbodiimide activation (several hours at room temperature or at 278 K). The procedure is further complicated by the difficulties of wetting the hydrophobic PDMS stamp with a polar buffer solution. This problem has been addressed by others by oxidizing the topmost layer of the PDMS stamp in a corona discharge or in an oxygen plasma.[61,62] This produces a thin hydrophilic silicate layer on the PDMS which, however, quickly loses its hydrophilicity and must be reactivated before each printing step. Very recently, an improvement could be demonstrated by chemically derivatizing an oxidized PDMS stamp with a poly(ethylene glycol) layer that allowed storing the stamps in air for at least 24 h with no loss in functionality for printing hydrophilic test solutions.[63] Using such a hydrophilic stamp together with the procedure outlined here should allow the formation of much better defined protein structures. Another strategy was proposed by Martin et al.[64] who prepared a hydrogel from 6-acryloyl-β-O-methylgalactopyranoside and either N,N'-methylenebisacrylamide or ethylene glycol dimethylacrylate cross-linkers. After swelling, the gel was mechanical robust to allow casting stamps from PDMS masters. The suitability for printing hydrophilic substances was tested by applying an aqueous dendrimer solution to the stamp and transferring it by physisorption to a polyvinylbenzyl chloride film where it was stained with a fluorescent dye. Because of the pore structure of the hydrogel, stamps made from

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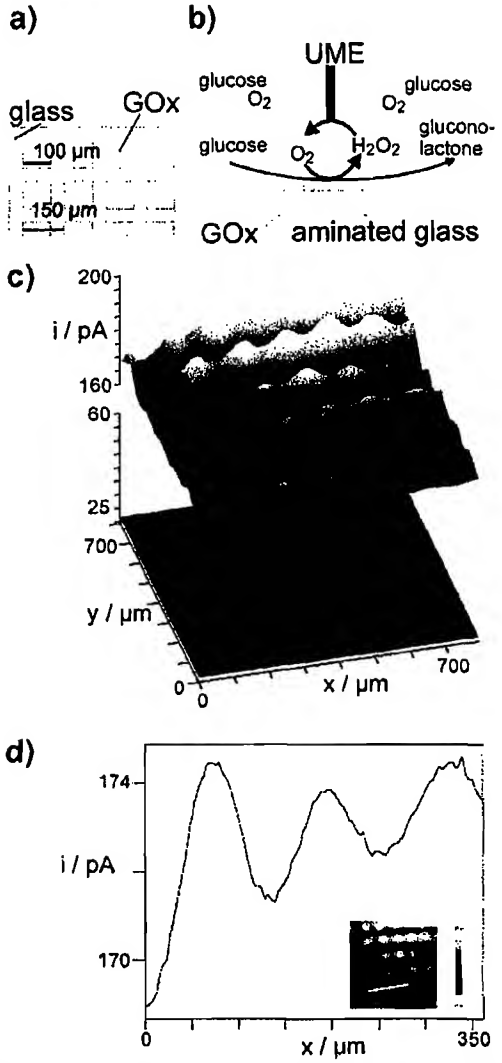

Figure 4. SECM imaging of localized GOx activity on patterned monolayers on glass. (a) A mixture of GOx and carbodiimide was applied to the stamp and brought into contact with an aminated glass surface on the 100 μm × 100 μm squares; contact time, 30 min. (b) Schematic of the SECM GC experiment. (c) Top image, air-saturated 50 mM glucose + phosphate buffer, pH 7; bottom image, air-saturated phosphate buffer without glucose, r_T = 12.5 μm, E_T = 750 mV. (d) Profile from (c).

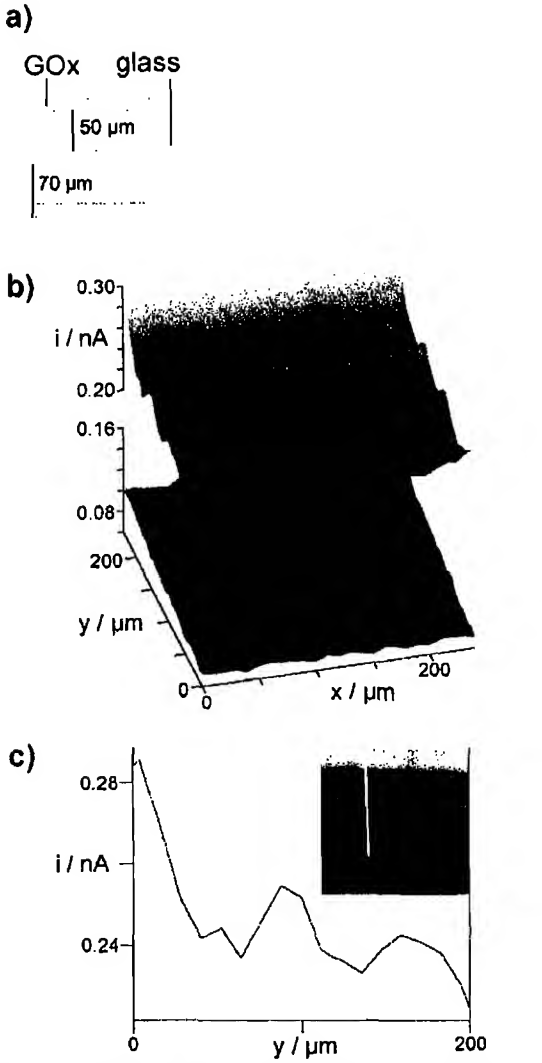

Figure 5. SECM imaging of carbodiimide-cross-linked GOx using MIMIC. (a) Layout of the pattern; the MIMIC channels are 20 μm wide and 10 μm deep, the pattern periodicity is 70 μm, and the incubation time is 2 h. (b) SECM GC imaging of activity (schematic in Figure 4b); top, air-saturated 50 mM glucose + phosphate buffer, pH 7; bottom, air-saturated phosphate buffer without glucose, r_T = 12.5 μm, E_T = 750 mV. (c) Profile from (b).

it should be able to accommodate protein molecules and allow multiple prints without reapplication of the protein solution.[34]

3.5. GOx Modification of Aminated Glass by MIMIC Using an Activating Agent. To avoid the harsh conditions during direct microcontact printing of a GOx–carbodiimide solution onto aminated glass, the same solution was incubated in the channels of a PDMS stamp similar to the concepts of MIMIC.[28] Here the enzyme can be kept in a buffered solution during the entire incubation period. Since the channels of the stamp are 10 μm deep, there is enough volume for the formation of a multilayer enzyme gel below the recessed channels of the stamp (Figure 5a). Therefore, higher activities of the immobilized enzyme layers are expected. This is confirmed by the SECM images recorded in the GC mode (Figure 5b) equal to those in Figure 4. The currents are higher by a factor of 2 (Figure 5b, upper image). However, there is almost no contrast between the regions of the MIMIC channel and the contact area. Most likely, the stamp failed to prevent the penetration of the solution between the hydrophilic glass surface and the stamp, and essentially the entire surface was modified with GOx. As described in the discussion of the direct stamping of GOx on glass, a considerable improvement of this strategy might be expected when using PDMS stamps with a hydrophilic surface modification. However, to maintain a good seal while having a hydrophilic channel, it is advantageous to render only the recessed areas hydrophilic.[65]

The decreased currents in Figure 5b, upper image, parallel to the low-frequency scan axis at y = 50, 100, 150,

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and 200 μm are artifacts resulting from electromagnetic interferences caused by holding currents of our positioning system and become visible at picoampere current levels. They are evident as artifacts because they occur with the same signal difference in the control experiment without glucose in solution (Figure 5b, lower image) whereas signals caused by the enzymatic activity of the sample decrease or vanish if the concentration of the enzyme substrate is decreased by solution exchange in the SECM cell.

Although not successful here, the MIMIC approach has proved to be very promising in similar but not identical situations. Patterns of different antibodies or of streptavidin could be formed by delivering the biomolecules to activated gold, glass, silicon, and polymer supports though a microfluidic network made from hydrophilized PDMS with about 3 μm wide channels.[9,10,65] The patterns retained their specific binding properties. The approach is potentially useful to form monolayers of a large variety of enzymes that may be deactivated by cross-linking with carbodiimide as a large variety of surface functionalities has been described.[66] Forming three-dimensional gel pads can be desirable in order to increase the enzyme activity per surface area. Formation of enzyme-loaded hydrogels within the MIMIC setup might be a promising perspective that would be applicable to a large variety of enzymes sensitive to covalent modifications by cross-linkers.

4. Conclusion

Different procedures have to be tested to form patterned enzyme layers on glass and gold. The local activity of the immobilized enzyme layers was measured by the GC mode of the SECM. Patterning of the gold surfaces leads to acceptable results for different procedures. Patterns of high activity and edge definition can be achieved by covalent coupling of the enzyme to amino groups of a patterned monolayer consisting of methyl-terminated and amino-terminated thiolates. High local enzyme activity was also found if a thiol group was introduced into horseradish peroxidase and the thiolated enzyme was directly chemisorbed onto bare gold regions. This procedure eliminates one incubation step in the formation of patterned protein layers and can therefore be very advantageous for a multistep procedure aiming to construct multienzyme patterns. Soft-lithographic approaches to create patterned enzyme layers on glass were less successful. Direct stamping of a dried mixture of glucose oxidase and carbodiimide gave patterns with low overall activity and low contrast most likely because the enzyme lost its activity when dried at room temperature and incubated for 30 min. Confinement of the surface functionalization by delivering the solution with enzyme and carbodiimide through a channel formed by the specimen surface and the side walls of a recessed line in a PDMS block (similar to MIMIC) did not yield a good contrast within the pattern most likely because the reaction mixture could creep between the aminated (hydrophilic) glass surface and the stamp during the incubation time of 2 h. Therefore, our further attempts to form multienzyme patterns for further local modification will make use of gold supports.

Acknowledgment. The work was supported by the Deutsche Forschungsgemeinschaft under Grant Number Wi 1617/1-3. T.W. was a grantee and associated member of the Graduate Course "Physical Chemistry of Surfaces and Interfaces" sponsored by the Deutsche Forschungsgemeinschaft.

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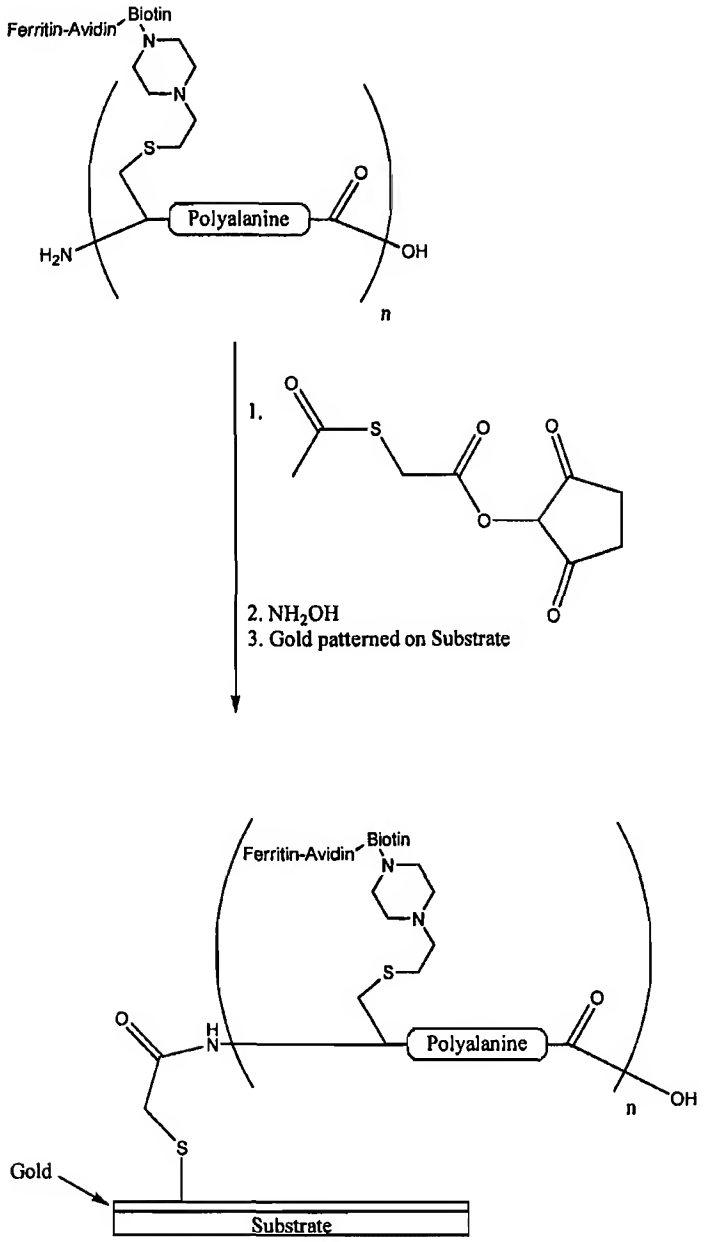
EXHIBIT E

Figure 2:


Ferritin-Avidin
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3. Gold patterned on Substrate
Ferritin-Avidin
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H
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Polyalanine
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Substrate

EXHIBIT F

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Carbon Nanotubes: Synthesis, Integration, and Properties

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ABSTRACT
Synthesis of carbon nanotubes by chemical vapor deposition over patterned catalyst arrays leads to nanotubes grown from specific sites on surfaces. The growth directions of the nanotubes can be controlled by van der Waals self-assembly forces and applied electric fields. The patterned growth approach is feasible with discrete catalytic nanoparticles and scalable on large wafers for massive arrays of novel nanowires. Controlled synthesis of nanotubes opens up exciting opportunities in nanoscience and nanotechnology, including electrical, mechanical, and electromechanical properties and devices, chemical functionalization, surface chemistry and photochemistry, molecular sensors, and interfacing with soft biological systems.

Introduction

Carbon nanotubes represent one of the best examples of novel nanostructures derived by bottom-up chemical synthesis approaches. Nanotubes have the simplest chemical composition and atomic bonding configuration but exhibit perhaps the most extreme diversity and richness among nanomaterials in structures and structure−property relations.[1] A single-walled nanotube (SWNT) is formed by rolling a sheet of graphene into a cylinder along an (m,n) lattice vector in the graphene plane (Figure 1). The (m,n) indices determine the diameter and chirality, which are key parameters of a nanotube. Depending on the chirality (the chiral angle between hexagons and the tube axis), SWNTs can be either metals or semiconductors, with band gaps that are relatively large (~0.5 eV for typical diameter of 1.5 nm) or small (~10 meV), even if they have nearly identical diameters.[1] For same-chirality semiconducting nanotubes, the band gap is inversely proportional to the diameter. Thus, there are infinite possibilities in the type of carbon tube "molecules", and each nanotube could exhibit distinct physical properties.

The past decade has witnessed intensive theoretical and experimental effort toward elucidating the extreme sensitivity of the electronic properties of nanotubes to their atomic structures.[2−6] It has been revealed that metallic and semiconducting nanotubes exist in all materials synthesized by arc-discharge, laser ablation, and chemical vapor deposition methods. Metallic SWNTs have become model systems for investigating the rich quantum phenomena in quasi-1d solids, including single-electron charging, Luttinger liquid, weak localization, ballistic transport, and quantum interference.[2−4,7,8] On the other hand, semiconducting nanotubes have been exploited as novel building blocks for nanoelectronics, including transistors and logic, memory, and sensory devices.[2−6]

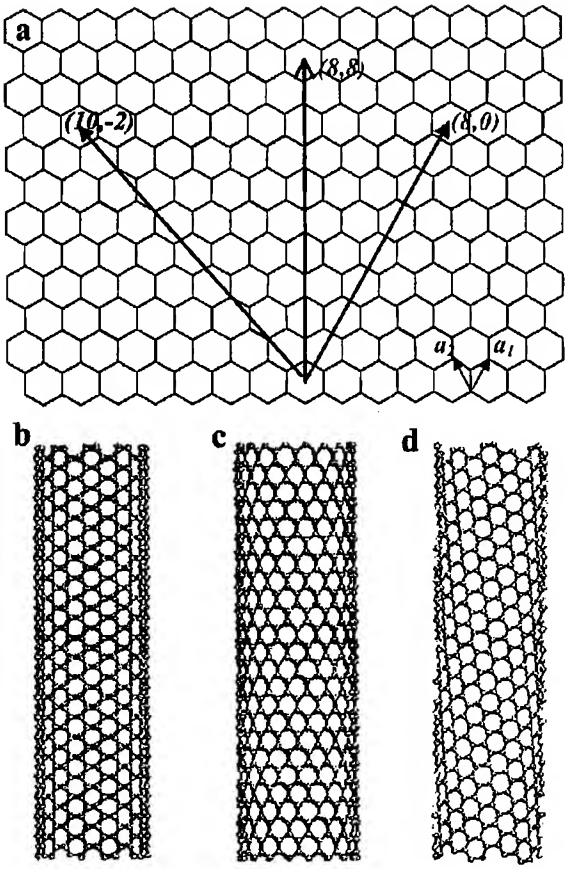

FIGURE 1. (a) Schematic honeycomb structure of a graphene sheet. Single-walled carbon nanotubes can be formed by folding the sheet along lattice vectors. The two basis vectors a_1 and a_2 are shown. Folding of the (8,8), (8,0), and (10,−2) vectors lead to armchair (b), zigzag (c), and chiral (d) tubes, respectively.

Nanotube characterization and device explorations have been greatly facilitated by progress in nanotube synthesis over the years. At the same time, many aspects of basic research and practical application requirements have been driving and motivating synthetic methods for better and more homogeneous materials. This complementary cycle continues, and it is now at a time when nanotube synthesis has evolved from enabling growth into consciously controlling the growth. While the extreme sensitivity of nanotube structure−property relations has led to rich science and promises a wide range of applications, it poses a significant challenge to chemical synthesis in controlling the nanotube diameter and chirality. Understanding how to synthesize nanotubes with predictable

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properties essentially requires exquisite control of atomic arrangement along the tubes, which is an ultimate task for chemists in the nanotube field.

Arc-discharge, laser ablation, and chemical vapor deposition have been the three main methods used for carbon nanotube synthesis.[6] The first two employ solid-state carbon precursors to provide carbon sources needed for nanotube growth and involve carbon vaporization at high temperatures (thousands of degrees Celsius). These methods are well established in producing high-quality and nearly perfect nanotube structures, despite large amounts of byproducts associated with them. Chemical vapor deposition (CVD) utilizes hydrocarbon gases as sources for carbon atoms and metal catalyst particles as "seeds" for nanotube growth that takes place at relatively lower temperatures (500−1000 °C).[6] For SWNTs, none of the three synthesis methods has yielded bulk materials with homogeneous diameters and chirality thus far. Nonetheless, arc-discharge and laser ablation techniques have produced SWNTs with impressively narrow diameter distributions averaging ~1.4 nm. CVD methods have come a long way from producing carbon fibers, filaments, and multiwalled carbon nanotubes to the synthesis of SWNTs[6,9−14] with high crystallinity and perfection comparable to those of arc[15] and laser[16] materials, as revealed by electrical transport and microscopy and spectroscopy measurements.

This Account presents our up-to-date research on controlling the growth of carbon nanotubes by CVD approaches. While arc-discharge and laser ablation methods produce only tangled nanotubes mixed randomly with byproducts, we illustrate that site-selective CVD synthesis on catalytic patterned substrates grows nanotube arrays *at controllable locations and with desired orientations* on surfaces. We further show successful chemical vapor deposition of SWNTs on preformed discrete catalytic nanoparticles. The results suggest that understanding the chemistry involved in the growth and controlling the catalytic nanoparticles could eventually allow for the control of the diameter and chirality of nanotubes. We also review elucidations of the interplay of electrical, mechanical, and chemical properties of nanotubes as facilitated by our progress in controlled materials synthesis. Implications of nanotube functionalization, molecular sensors, and interfacing with biological molecules are discussed.

Patterned Growth of Nanotubes

Carbon nanotube synthesis by CVD involves heating a catalyst material in a furnace and flowing a hydrocarbon gas through the tube reactor for a period of time. The catalytic species are transition-metal nanoparticles typically supported on high surface area materials (alumina) materials.[6] Simplistically, the catalyst particles serve as seeds to nucleate the growth of nanotubes. We have developed patterned growth approaches to obtain organized nanotube structures. The idea is to position catalyst in arrayed fashions for the growth of nanotubes from specific catalytic sites on surfaces.[9] We have carried out such patterned growth for both multiwalled and single-walled nanotubes, exploited ways including self-assembly and active electric field control to manipulate the orientation of nanotubes, and pursued several generations of catalysts ranging from powdery supported catalyst to discrete catalytic nanoparticles. These works have led to ordered nanotube arrays or networks formed at the synthesis stage of nanotubes.

(a) Ordered Arrays of Multiwalled Nanotubes—Self-Assembly by Intratube van der Waals Interactions. Our earlier work has demonstrated the synthesis of regular arrays of ordered towers consisting of multiwalled nanotubes (MWNTs) by CVD growth (700 °C; carbon source, C_2H_4; alumina-supported iron catalyst) on porous silicon or silicon substrates patterned with iron particles in square regions (Figure 2a).[17] The nanotubes within each tower are well aligned along the direction perpendicular to the substrate surface. The alignment is a result of nanotubes grown from closely spaced catalyst particles in each square self-assembling into rigid bundle structures due to strong intratube van der Waals binding interactions. The rigidity of the assembly allows the nanotubes to self-orient and grow perpendicular to the substrate. The arrayed nanotubes exhibit excellent characteristics in electron field emission, opening up the possibility of spatially defined massive field emitter arrays derived by simple chemical routes for flat panel display applications.[17]

(b) Ordered Networks of Suspended Single-Walled Nanotubes—Self-Assembly by van der Waals Interactions with Substrates. We have synthesized suspended SWNT networks with well-defined orientations on substrates containing lithographically patterned silicon posts.[18,19] Contact printing is used to transfer catalyst materials onto the tops of pillars selectively, and CVD (900 °C; carbon source, CH_4; supported iron catalyst derived by a template method from liquid precursors) on the substrates leads to suspended SWNTs forming nearly ordered networks with the nanotube orientations directed by the pattern of the silicon posts (Figure 2b−d). The mechanism for nanotube self-orienting in this case is due to van der Waals interactions between nanotubes and the silicon posts. The nanotubes grown from the posts float and wave in the gas until they are fixed by nearby posts. The suspended nanotube networks are difficult to obtain otherwise by, e.g., postgrowth assembly with arc- or laser-grown materials and are useful for building novel nanoelectromechanical devices (NEMs).

(c) Electric-Field-Directed Nanotube Growth. We recently have exploited electric fields to actively control the growth directions of SWNTs.[20] Highly aligned suspended SWNTs are grown under electric fields on the order of ~1 V/μm (Figure 2e). The alignment effect originates from the high polarizability of SWNTs. The induced dipole moment for a ~10 μm SWNT in a 1 V/μm electric field is about 10^6 D, leading to a large aligning torque that directs the nanotube parallel to the electric field. Importantly, the electric field alignment effect is stable against thermal fluctuations at the growth temperature and against gas

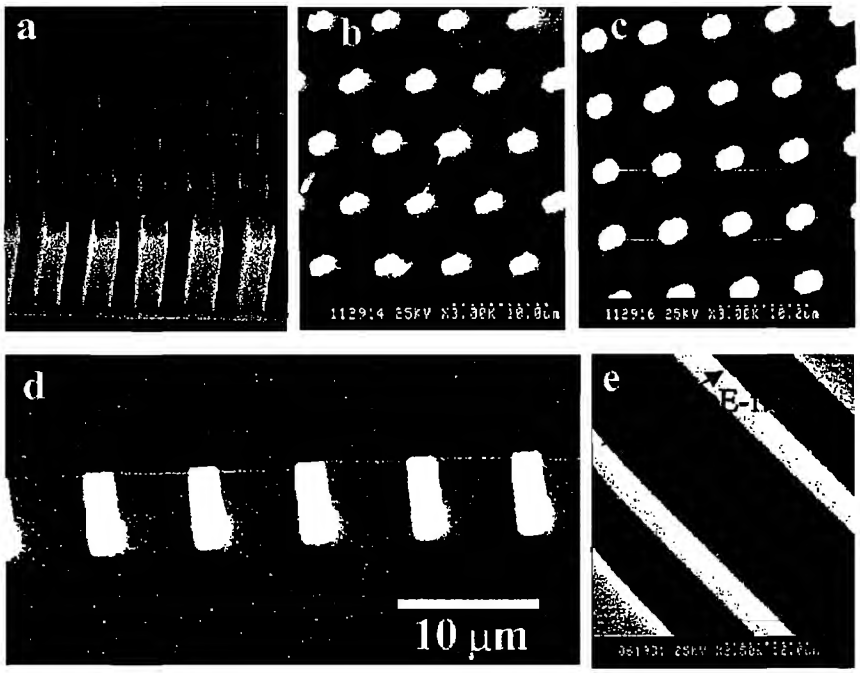

FIGURE 2. Ordered carbon nanotube structures obtained by direct chemical vapor deposition synthesis. (a) An SEM image of self-oriented MWNT arrays. Each tower-like structure is formed by many closely packed multiwalled nanotubes. Nanotubes in each tower are oriented perpendicular to the substrate. (b) SEM top view of a hexagonal network of SWNTs (line-like structures) suspended on top of silicon posts (bright dots). (c) SEM top view of a square network of suspended SWNTs. (d) Side view of a suspended SWNT power line on silicon posts (bright). (e) SWNTs suspended by silicon structures (bright regions). The nanotubes are aligned along the electric field direction.

flow randomization effects. Manipulation by applied fields during nanotube synthesis is highly promising for further exploration of both suspended molecular wires and complex nanotube fabric structures on flat substrates.

(d) From Powdery Catalyst to Isolated Catalytic Nanoparticles—Toward Nanotube Structural Control and Microscopic Understanding of Growth. Supported catalyst typically used for SWNTs growth consists of powdery support with attached metal nanoparticles that are difficult to characterize by microscopy. As a result, the size of the catalytic nanoparticles is hard to control. Recently, we have pursued isolated iron nanoparticles for the synthesis of SWNTs by CVD.[21,22] Controllable numbers of Fe^{3+} are placed into the cores of apoferritin to afford stable solutions of artificial ferritin that can be subsequently deposited onto flat substrates. Calcination in air leads to discrete Fe_2O_3 nanoparticles with average diameter ~1.5 nm, as characterized by atomic force microscopy (AFM) and transmission electron microscopy (TEM). AFM and TEM have revealed successful growth of SWNTs from the isolated nanoparticles (Figure 3). Further, synthesis of SWNTs directly on TEM grids has allowed us to clearly reveal the nanoparticle–nanotube relationship (Figure 3a–f).

Our data provide evidence that, first, the diameters of SWNTs are closely determined by the diameters of catalytic particles and second, TEM imaging of both ends of isolated SWNTs (Figure 3a,d,e) points to the so-called base-growth model of SWNTs in our CVD process. That is, the catalytic nanoparticle remains on the supporting substrate, and a nanotube grows out from the particle with a dome-closed end (Figure 3g). The CVD reaction process then involves carbon atoms, decomposed from methane, absorbing into the anchored nanoparticle on the substrate to form a carbon–iron solid-state solution. As supersaturation occurs, carbon atoms precipitate out from the particle, leading to the growth of a nanotube (Figure 3g). This growth model is in contrast with the tip-growth model, in which catalyst particles lift off from the substrate during growth.

Synthesis with controllable catalyst, although still in its early stage,[21–23] has led to important clues to the nanotube growth chemistry, mechanism, and structural control. Future work will include determining the nature of SWNTs (metal vs semiconductor) grown from individual particles, experimentally and theoretically investigating how a seed particle determines the nanotube chirality, and obtaining highly monodispersed nanoparticles at the 1 or 2 nm level. We have also carried out patterned growth with ferritin particles placed in micrometer-scale squares on surfaces (Figure 3h). New lithographic approaches capable of 10 nm features are required to enable catalytic patterning of individual nanoparticles. Meeting these challenges will be extremely rewarding, leading to (1) rational routes to nanotube diameter and chirality control and (2) dense and ordered networks of nanotubes with predictable metallic or semiconducting electronic properties.

(e) Scalability of Patterned Growth. Assembly of nanotube arrays must be scaled-up on large substrates for various practical applications. To this end, we have

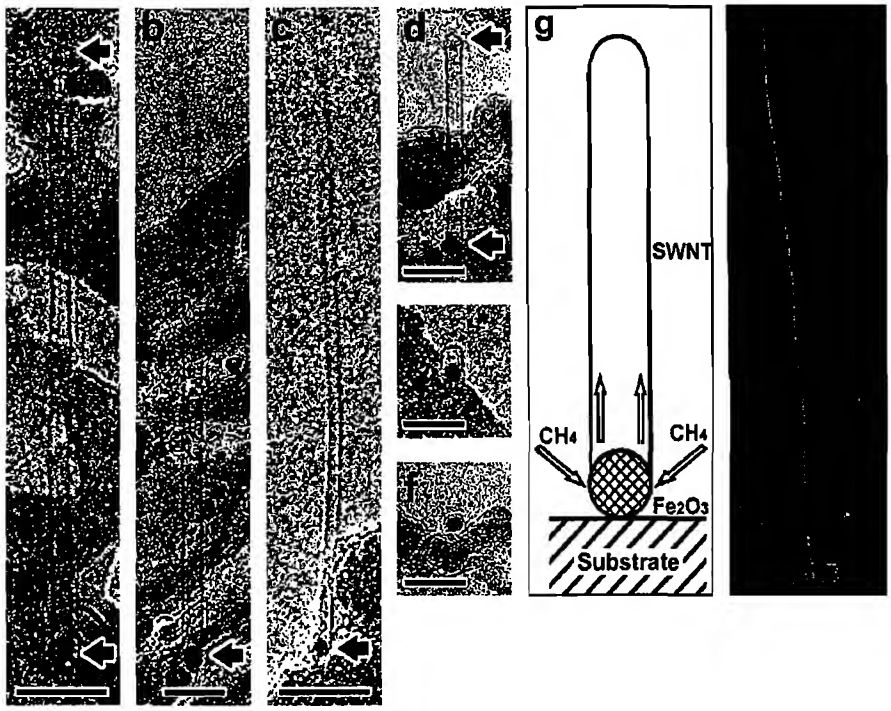

FIGURE 3. (a–f) TEM images of SWNTs grown from discrete nanoparticles, showing particle–nanotube relationships. Scale bars: 10 nm. (a–d) SWNTs grown from discrete nanoparticles (dark dots at the bottom of the images). The arrows point to the ends of the nanotubes. The ends extending out of the membrane in (b) and (c) are not imaged due to thermal vibration. The background roughness reflects the TEM grid morphology. (e) Image of an ultrashort (~4 nm) nanotube capsule grown from a ~2 nm nanoparticle. (f) Image of a nanoparticle surrounded by a single graphitic shell. (g) A schematic model for nanotube growth. (h) AFM image of a 50 μm long SWNT grown from nanoparticles patterned into the circled region.

explored patterned growth of SWNTs on full 4-in. SiO_2/Si wafers.[24] We have patterned micrometer-sized catalyst islands with high uniformity over entire wafers by a photolithography approach (Figure 4a). We have also investigated how the gas flow rates (CH_4 and H_2 co-flow) affect the growth of SWNTs. This aspect has not been systematically studied in our earlier synthesis work. We have identified that, for relatively low CH_4 flow rates, the CVD process is ultrasensitive to the amount of H_2 co-flow, undergoing pyrolysis, growth, and inactive reaction regimes with increased H_2 addition. This understanding has enabled us to grow high-quality SWNTs (Figure 4b) from massive arrays of well-defined surface sites on full 4-in. wafers.[24]

Electrical Properties and Interplay with Mechanical and Chemical Properties

Patterned growth of carbon nanotubes on substrates has allowed for convenient, simple, and controlled integration of nanotubes into various device structures for elucidating the properties of individual molecular wires. With these devices, we have carried out electron transport measurements of semiconducting, quasi-metallic, and metallic SWNTs grown by CVD. Suspended nanotubes have also been integrated into addressable structures for mechanical and electromechanical characterization.

(a) Electrical Properties of Nanotubes. We have revealed by transport measurements (Figure 5a) that the majority of individual SWNTs synthesized by CVD on supported catalyst are semiconducting in nature. This type of nanotube exhibits field effect transistor (FET) behavior at room temperature and has been intensively explored in recent years for nanoelectronics devices, including transistors and logic devices.[3,25–29] The as-grown semiconducting SWNTs are hole-doped p-type FETs with hole depletion and diminished conductance (from typically 100 kΩ to 1 MΩ) by positive gate voltages (Figure 5b). It is now established that molecular oxygen adsorbed on the nanotubes is responsible for hole doping of SWNTs.[30–33] Removal of O_2 can lead to nearly intrinsic semiconducting behavior (Figure 5b inset).[33] More and more investigations are revealing that, although carbon nanotubes are highly robust and inert structures, their electrical properties are extremely sensitive to charge transfer and chemical doping effects by various molecules.

The second type of observed SWNTs synthesized by CVD appears to be quasi-metallic with small band gaps on the order of 10 meV.[34] These nanotubes are not as sensitive as semiconducting SWNTs to electrostatic doping by gate voltages but exhibit a conductance dip associated with the small band gap. These nanotubes correspond to a class of non-armchair SWNTs, and the origin of the band

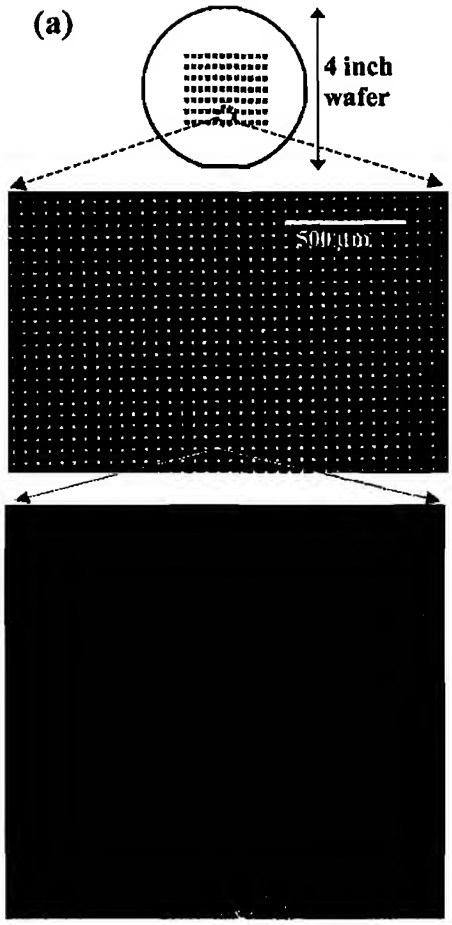

FIGURE 4. Wafer scale patterned nanotube synthesis. (a) An optical image showing patterned catalyst islands (bright dots). (b) AFM image of SWNTs grown and emanating from one of the catalyst islands on the wafer.

gap is slight sp^2 to sp^3 hybridization due to the nonflat nature of the hexagons on the tube walls.[35,36] Temperature-dependent measurements for some of the quasi-metallic-like SWNTs (room-temperature resistance ~10–20 kΩ) reveals increased electrical conductance at low temperatures, reaching the $4e^2/h = 2G_0$ quantum conductance (6.45 kΩ in resistance) limit at ~1.5 K (Figure 5c). Quantum interference effects have also been observed.[8] The results suggest that (1) phonon is one of the fundamental scattering mechanisms in SWNTs at room temperature, (2) excellent ohmic contacts can be made to nanotubes with transmission probability $T \approx 1$, and (3) electron transport is highly phase coherent and ballistic in nanotubes at low temperatures.[8]

Truly metallic armchair SWNTs are rare in our CVD-grown nanotubes. The conductance of this type of nanotube is the least sensitive to gate voltages. With highly transparent ohmic contacts to these nanotubes, we have observed near $4e^2/h$ quantum conductance in SWNTs as long as 4 μm at low temperatures (J. Kong and H. Dai, unpublished results), suggesting a long mean free path

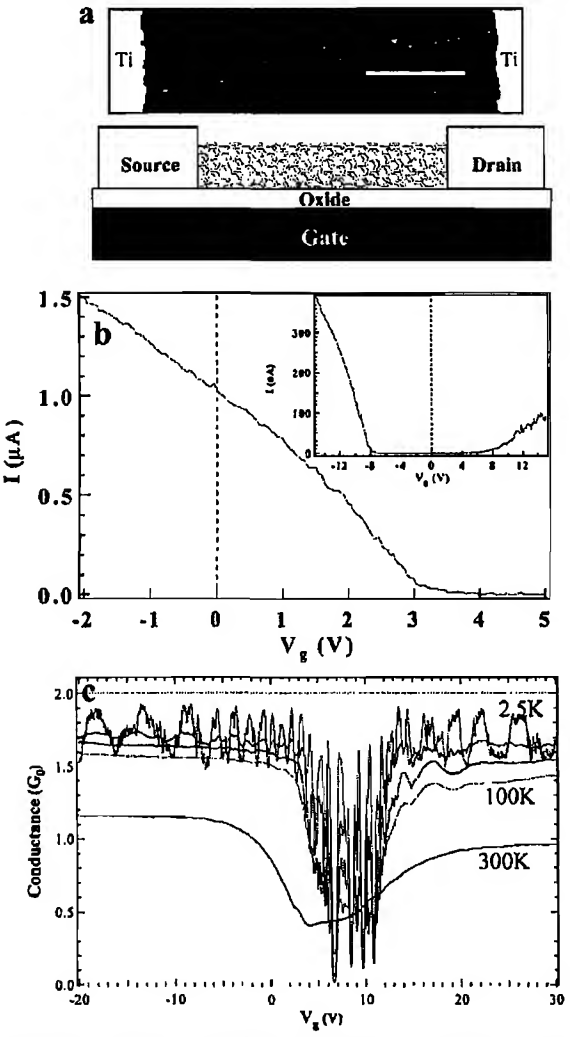

FIGURE 5. Electrical properties of individual nanotubes. (a) AFM image of a SWNT contacted by two Ti electrodes and a schematic cross-section view of the device. Scale bar: 1 μm. A gate voltage (V_g) can be applied to electrostatically couple to the nanotube and shift the Fermi level. (b) $I-V_g$ curve for a typical as-made semiconducting SWNT showing p-type FET characteristics. Inset: The p-type FET evolves into a nearly intrinsic semiconductor after removal of surface-adsorbed oxygen. (c) Conductance G (in units of quantum conductance, $G_0 = 2e^2/h$) vs V_g (Fermi energy) for a quasi-metallic SWNT at various temperatures. The conductance approaches the theoretical limit $2G_0$ at low temperatures, with conductance fluctuations due to quantum resonance effects.

for ballistic electron transport in high-quality/perfection CVD-grown SWNTs.

(b) Nanotube Electromechanical Properties and Devices. Patterned growth has been used to synthesize suspended SWNTs across trenches with the nanotubes wired up electrically with relative ease.[37] We have manipulated a suspended nanotube by using an AFM tip while monitoring its electrical conductance (Figure 6a,b). By so doing, we are able to address how mechanical deformation affects the electrical properties of carbon

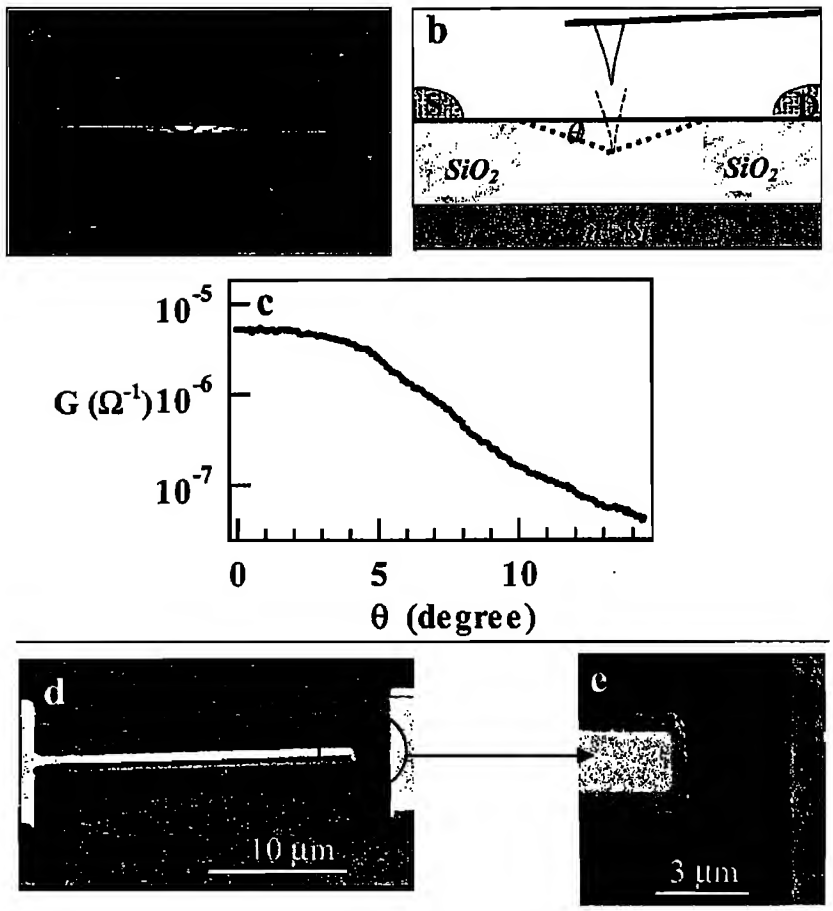

FIGURE 6. Nanoelectromechanics of suspended nanotubes. (a) AFM image of a suspended SWNT over a trench (dark region, ~500 nm wide). (b) Experimental scheme for measuring the electromechanical property of the nanotube. The suspended nanotube is pushed by an AFM tip, while the conductance of the nanotube between the source (S) and drain (D) electrodes is monitored. (c) Conductance *G* of the nanotube vs bending angle. (d,e) SEM images showing a suspended nanotube bridging a micromechanical Si beam and a Si terrace.

nanotubes, a question important to potential nanoelectromechanical (NEM) devices.

We have measured conductance vs strain and bending angle of individual SWNTs and found a 2 orders of magnitude conductance decrease at a 14° bending angle and 3% strain (Figure 6c). The electrical and mechanical changes are fully reversible, suggesting potential nanowire-based electromechanical transducers.[37] The electromechanical behavior is rationalized by molecular dynamics simulation and conductance calculations. Large local structural distortion of the nanotube caused by mechanical actions of the AFM tip leads to carbon atoms in sp^3-like bonding configurations, which is responsible for electron localization and significant local barriers for electron transport through the nanotube.[37,38]

We are extending our patterned growth approaches to obtaining new types of suspended nanotube NEMs structures for basic characterization and potential devices. Suspended SWNTs have been integrated with silicon-based micromechanical structures, as shown in Figure 6, with SEM images of suspended SWNTs grown on a suspended silicon beam and bridging a terrace (Figure 6d,e). Along this line, a wide range of nanotube-based NEMs structures are obtainable for exploring pure stretching, twisting of single nanowires, and their high-frequency resonance characteristics. Functional NEMs switches and memory devices can also be envisioned. Controlled and deterministic synthesis of nanotubes will continue to open exciting and new possibilities of novel nanostructures and devices.

Interactions with Chemical Species—From Small Molecules to Polymers

Single-walled carbon nanotubes are typically chemically inert. Covalent attachment of molecular species to fully sp^2-bonded carbon atoms on the nanotube sidewalls proves to be difficult. Adsorbing molecules to nanotubes via noncovalent forces, however, turns out to be facile and has important consequences to their physical properties and potential applications.

(a) Molecular Adsorption and Photodesorption. The first sign of molecular adsorption on SWNTs was noticed during electrical transport measurements as the resistance

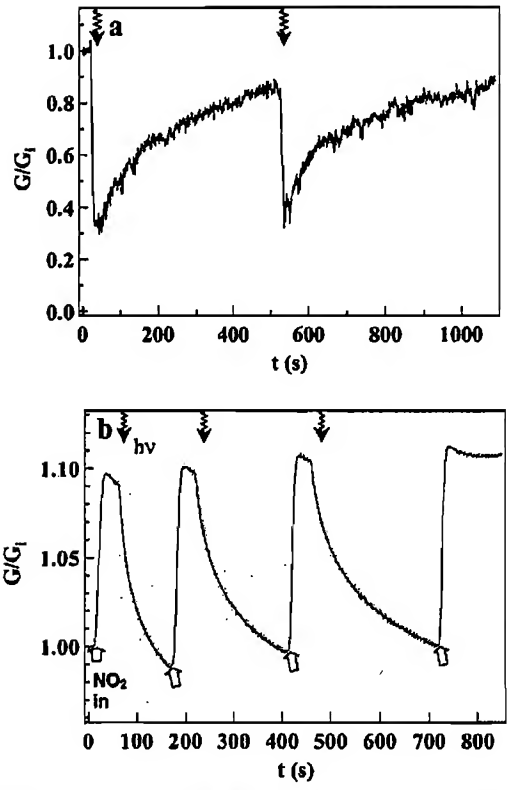

FIGURE 7. Molecular adsorption, photodesorption, and reversible chemical sensors. (a) Normalized conductance of an individual semiconducting SWNT during O_2 desorption caused by UV illumination (shaded time periods) and O_2 re-adsorption after turning off the UV. (b) Conductance response of an ensemble of SWNTs to NO_2 adsorption and photodesorption (during shaded time periods) cycles. The wide arrows point to the times when NO_2 is introduced into the measurement chamber and adsorbs onto SWNTs.

of semiconducting SWNTs tended to change in a vacuum versus that in air. We have systematically measured the electrical properties of SWNTs in various chemical environments and revealed various small gas molecules adsorbing onto SWNTs and undergoing charge transfer.[30] As a result, semiconducting SWNTs exposed to ppm levels of NO_2 exhibit conductance increases by up to 3 orders of magnitude in a few seconds. When the SWNTs are exposed to NH_3, the conductance of the nanotube can decrease by up to 2 orders of magnitude.[30] Collins and co-workers have found similar high sensitivity to O_2 adsorption.[31] These results have suggested nanotubes as miniature chemical sensors with high sensitivity at room temperature.

NO_2 and O_2 on SWNTs are strongly physisorbed and do not desorb at significant rates at room temperature. NH_3 is weakly physisorbed on nanotubes and can be removed by simple pumping in a vacuum. Density functional theory (DFT) calculations reveal that NO_2 binds to a nanotube with an energy on the order of ~0.4 eV[39] to 0.9 eV[30,40] and withdraws ~e/10 from the nanotube. O_2 binds to a nanotube with ~0.25 eV energy and ~e/10 charge-withdraw from the tube.[41] However, DFT calculation does not reveal any significant binding of NH_3 on nanotubes, and NH_3 appears to be weakly adsorbed on nanotubes by van der Waals interactions. Despite very small charge transfer, NH_3 adsorption does cause appreciable changes to the electrical property of SWNTs observed experimentally, suggesting the extreme sensitivity of SWNTs to chemical gating effects.

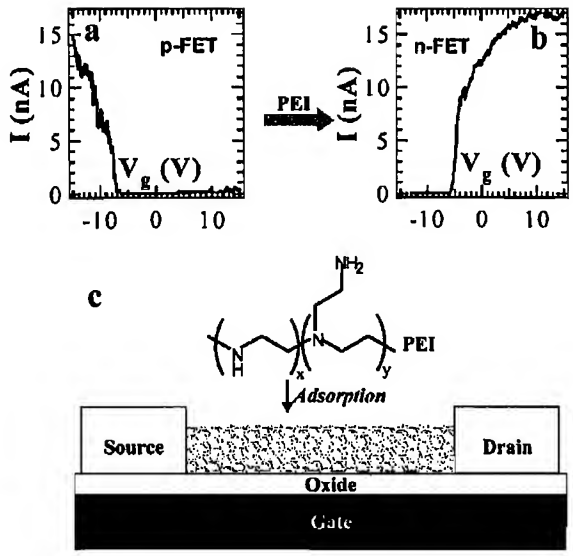

FIGURE 8. Polymer functionalization for air-stable n-type nanotube FET. (a) Current (I) vs gate characteristics of an as-made semiconducting SWNT showing p-type FET behavior. (b) The nanotube evolves into an n-type FET after adsorption of PEI, schematically shown in (c).

We have recently discovered photoinduced molecular desorption from SWNTs.[33] Desorption of molecules from SWNTs can certainly by achieved by heating nanotubes to high temperatures (~200 °C).[31,32] Ultraviolet (UV, 250 nm) light illumination at low photon flux causes rapid molecular desorption from SWNTs at room temperature. Figure 7a shows photodesorption of O_2 and thus lowered hole-carriers in a nanotube, as signaled by the rapid electrical conductance decrease upon UV illumination. Upon turning off the UV, conductance recovery occurs due to O_2 readsorption (Figure 7a). The photodesorption phenomenon is found to be generic to various molecules (Figure 7b) preadsorbed onto nanotubes.[33]

Wavelength-dependent measurements[33] reveal that photodesorption is due to electronic excitation of nanotubes and is a nonthermal process. Electronic excitations of π-plasmons in SWNTs by UV leads to electron/hole pairs via Landau damping.[42] We have suggested that the electrons or holes generated may attach to adsorbed molecular species, which is responsible for the observed molecular desorption. The photodesorption cross section is estimated to be $\sigma \approx 1.4 \times 10^{-17}$ cm^2 at 250 nm for O_2.[33]

Our results illustrate that surface chemistry and photochemistry issues are critical to the properties and applications of molecular-scale wires that have ultrahigh surface area, with every atom on the surface. Surface

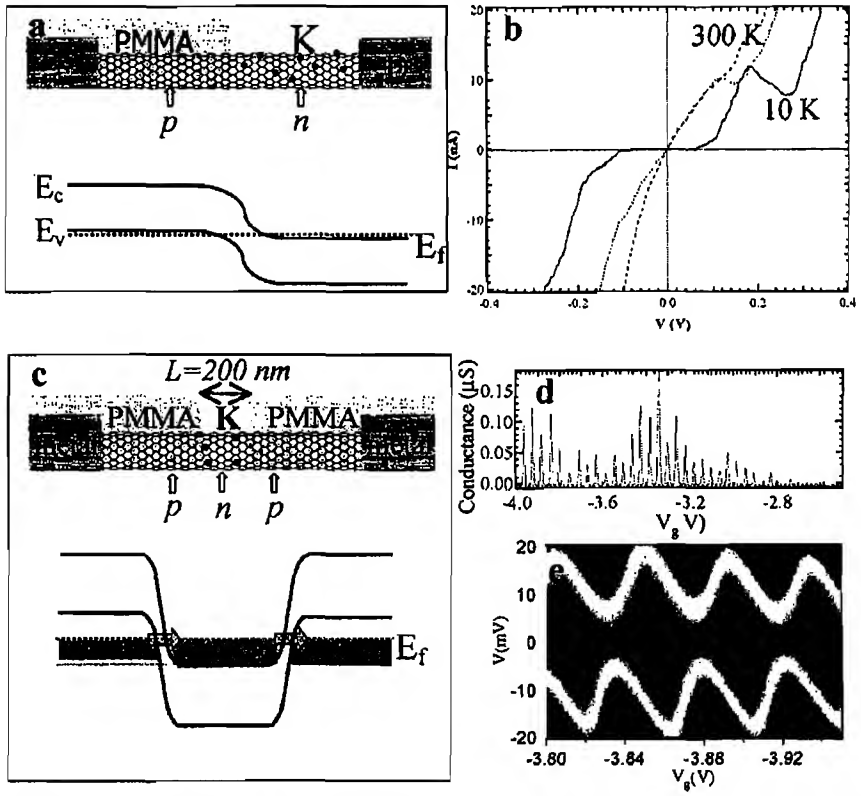

FIGURE 9. Chemical profiling of individual nanotubes. (a) Schematic structure and band diagram for a p−n junction formed on a nanotube. (b) Current−voltage ($I-V$) characteristics of the sample showing negative differential conductance at low temperatures. (c) Schematic structure and band (E_c, E_v, and E_f are conduction band, valence band, and Fermi level, respectively) diagram for two p−n junctions formed on a nanotube. (d) Periodic conductance oscillations vs gate voltage corresponding to single-electron transport. (e) Coulomb diamonds related to single-electron transport. The diamonds enclose regions with low conductance. The number of electrons in the quantum dot changes by 1 between adjacent diamonds.

science can be elucidated at the single-wire level by using electrical/chemical properties of nanotubes as probes.

(b) Polymer Doping. Controlling the type of charge carriers (electron or hole) is critical to conventional n-type or p-type FETs. By the same token, both p- and n-type FETs based on SWNTs are highly desired for potential nanoelectronics. However, the classical alkali metal n-dopants for carbon systems readily oxidize and are undesirable for practical nanotube n-FETs. We have carried out functionalization of SWNTs by an amine-rich polymer, *polyethyleneimine* (PEI), and obtained *n-type* FETs that are stable in air.[43] An as-made p-type semiconducting SWNT due to adsorbed O_2 readily reverts to an n-type FET after functionalization by PEI (Figure 8). Analysis shows that the adsorbed PEI donates approximately 1 electron to every 1000 carbon atoms on the nanotube.[43] Hence, instead of complete ionization of alkali, partial donating or withdrawing functional groups on organic materials can be used to dope and manipulate the charge carried in molecular wires, which may prove useful for nanoelectronics applications.

(c) Chemical Profiling of a Single Nanotube. We have shown that doping a nanotube with a controlled chemical dopant profile along its length leads to interesting quantum phenomena.[44-46] Compared to the molecular-scale diameters (1−2 nm), the lengths of SWNTs are mesoscopic or macroscopic (1−100 μm) and have ample room to form nanometer junctions. We have masked half of a SWNT by polymer and doped the other half by potassium to form an intratube p−n junction (Figure 9a) that exhibits negative differential conductance (NDC) at low temperatures (Figure 9b).[44] NDC arises due to quantum tunneling across degenerately doped semiconducting p−n junctions, known in conventional Esaki diodes. Nondegenerate intratube p−n junctions have been formed on SWNTs by organic amine doping, leading to excellent intratube electrical rectifiers.[45]

We have extended the scheme to chemically profile a nanotube containing two p−n−p junctions (Figure 9c).[46] Electron transport measurements reveal that the nanotube section between the two chemically formed junctions defines a small (~200 nm long) quantum dot on a micrometers-long nanotube. The small dot on the long tube exhibits clear single-electron charging phenomenon (Figure 9b),[47] as a result of small structural dimensions and hence low capacitance and high charging energy

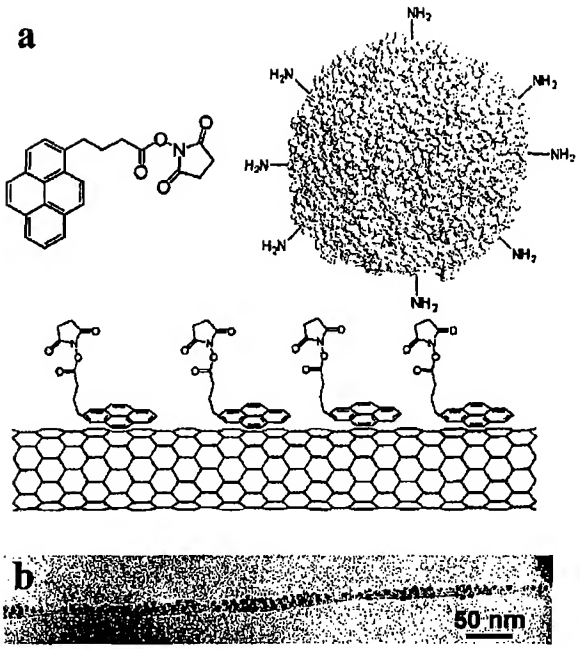

FIGURE 10. (a) Scheme for noncovalent functionalization of the sidewalls of nanotubes for protein immobilization. (b) A TEM image showing ferritin immobilized on a suspended nanotube by using the scheme in (a).

($>K_BT$) for the "dot". Controlled chemical doping can hence lead to interesting quantum phenomena and devices confined along the length of a nanotube.

Functionalization and Interfacing with Biological Systems

Functionalization is an important aspect of the chemistry of nanotube "macromolecules".[48-52] As mentioned, however, covalent sidewall functionalization of nanotubes from sp^2 to sp^3 structure is both difficult and undesired because of the loss of conjugation.[49] Alternatively, it is possible to functionalize SWNTs noncovalently[50-52] to preserve the sp^2 structures and their electronic properties that are useful for various postfunctionalization applications.

We have developed a simple and general approach to noncovalent functionalization and subsequent immobilization of various biological molecules onto nanotubes.[52] The noncovalent scheme involves π-stacking of 1-pyrenebutanoic acid succinimidyl ester onto the sidewalls of SWNTs (Figure 10a). The anchored pyrene moieties on SWNTs are highly stable against desorption in aqueous solutions, leading to functionalization of SWNTs with succinimidyl ester groups. The mechanism of protein immobilization on nanotubes, then, involves the nucleophilic substitution of *N*-hydroxysuccinimide by an amine group on the protein, resulting in the formation of an amide bond. This technique has enabled the immobilization of a wide range of biomolecules on the sidewalls of SWNTs with high efficiency, as demonstrated with ferritin (Figure 10b), streptavidin, and biotin-PEO-amine.[52]

Nanotube functionalization and bioimmobilization are motivated by the recent activities in biological applications of novel solid-state nanomaterials. The unique physical properties of molecular-scale or nanoscale solids (dots or wires), when utilized in conjunction with the remarkable biomolecular recognition capabilities, could lead to miniature biological electronic devices, including probes and sensors. The interface between biological molecules and nanomaterials is critical to such applications. With nanotubes, such exploration is still in its early stage, with wide-open room and many possibilities.

Summary

This Account has presented our work from controlling the synthesis of carbon nanotubes to using them as model systems for nanoscale science. The topics range from materials chemistry, solid-state physics, and surface chemistry/photochemistry to biological aspects of nanomaterials. As the synthesis–characterization–application cycle continues for nanotubes, gaining further control over their synthesis will remain at the heart of our research. Further exciting developments in nanoscale science and technology are expected.

This work was carried out by a group of outstanding students and postdoctoral fellows. We have greatly enjoyed collaborations with Professors C. Quate, S. Fan, S. Y. Wu, C. S Jayanthi, K. Cho, S. Manalis, C. Marcus, and R. Laughlin. This work has been supported by NSF, DARPA, SRC, the Packard Foundation, the Sloan Foundation, ABB, a Terman Fellowship, NSF/NNUN, LAM (Stanford), the Camile Henry Dreyfus Foundation, and the ACS-PRF.

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EXHIBIT G

US006548255B2

(12) **United States Patent**
Bensimon et al.

(10) **Patent No.: US 6,548,255 B2**
(45) **Date of Patent: *Apr. 15, 2003**

(54) **MOLECULAR COMBING PROCESS FOR DETECTING MACROMOLECULES**

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(73) Assignee: **Institut Pasteur and Centre National de la Recherche Scientifique**, Paris (FR)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/879,818**

(22) Filed: **Jun. 13, 2001**

(65) **Prior Publication Data**

US 2002/0031774 A1 Mar. 14, 2002

Related U.S. Application Data

(63) Continuation of application No. 08/467,529, filed on Jun. 6, 1995, now Pat. No. 6,294,324, which is a continuation of application No. 08/386,485, filed on Feb. 10, 1995, now Pat. No. 6,265,153.

(30) **Foreign Application Priority Data**

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Jun. 17, 1994 (FR) 94 07444

(51) **Int. Cl.**[7] **C12Q 1/68**; C07M 21/02; G01N 33/53

(52) **U.S. Cl.** 435/6; 435/7.1; 435/91.2; 435/4; 536/22.1; 536/23.1; 536/24.3; 530/350; 530/387.1

(58) **Field of Search** 435/6, 7.14, 91.2; 536/22.1, 23.1, 24.3; 530/350, 387.1

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Primary Examiner—Jeffrey Fredman
(74) *Attorney, Agent, or Firm*—Finnegan, Henderson, Farabow, Garrett & Dunner, L.L.P.

(57) **ABSTRACT**

The subject of the present invention is a process for aligning a macromolecule (macromolecules) on the surface S of a support, characterized in that the triple line S/A/B (meniscus) resulting from the contact between a solvent A and the surface S and a medium B is caused to move on the said surface S, the said macromolecules having a part, especially an end, anchored on the surface S, the other part, especially the other end, being in solution in the solvent A.

The subject of the present invention is also a process for detecting, measuring the intramolecular distance of, separating and/or assaying a macromolecule in a sample in which a process of alignment according to the invention is used.

54 Claims, 10 Drawing Sheets

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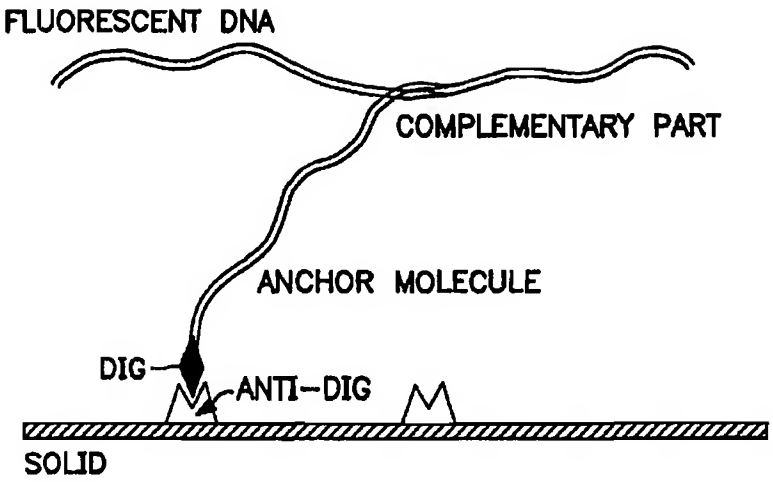
FLUORESCENT DNA
COMPLEMENTARY PART
ANCHOR MOLECULE
DIG
ANTI-DIG
SOLID

FIG. 1

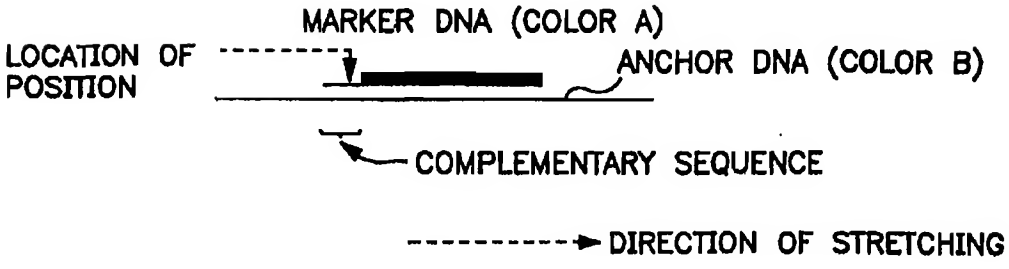
MARKER DNA (COLOR A)
LOCATION OF POSITION
ANCHOR DNA (COLOR B)
COMPLEMENTARY SEQUENCE
DIRECTION OF STRETCHING

FIG. 2

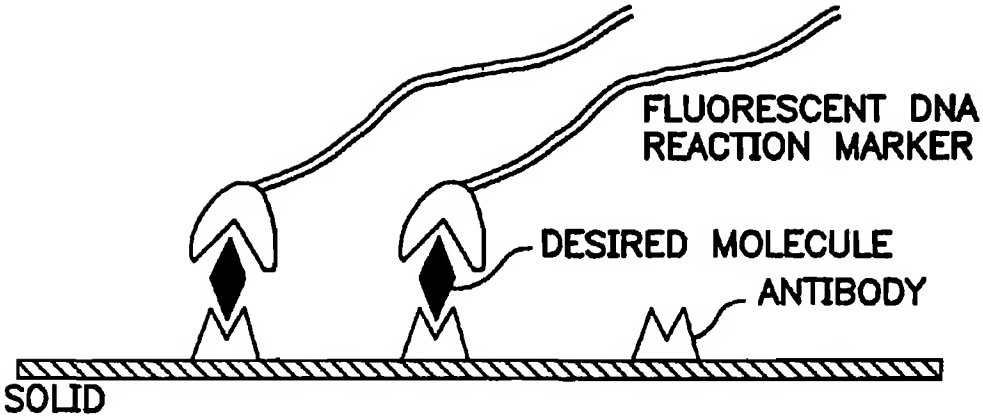
FLUORESCENT DNA
REACTION MARKER
DESIRED MOLECULE
ANTIBODY
SOLID

FIG. 3

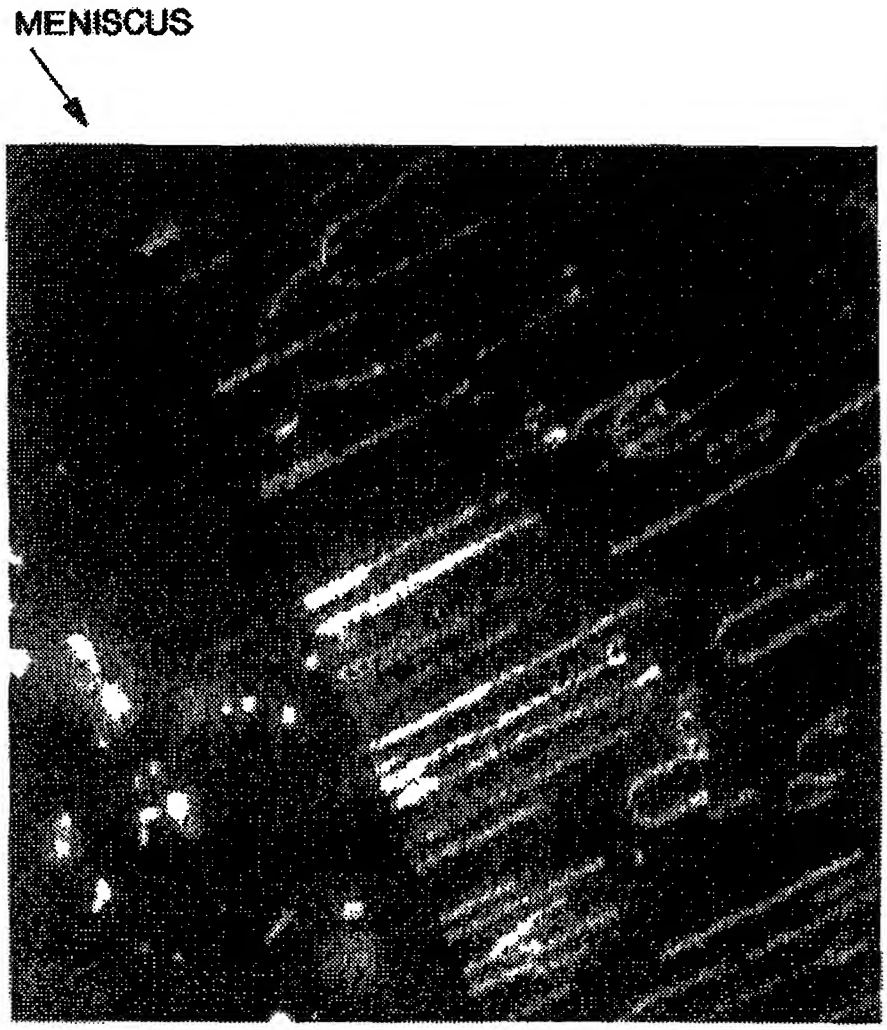

FIG. 4

FIG. 5a

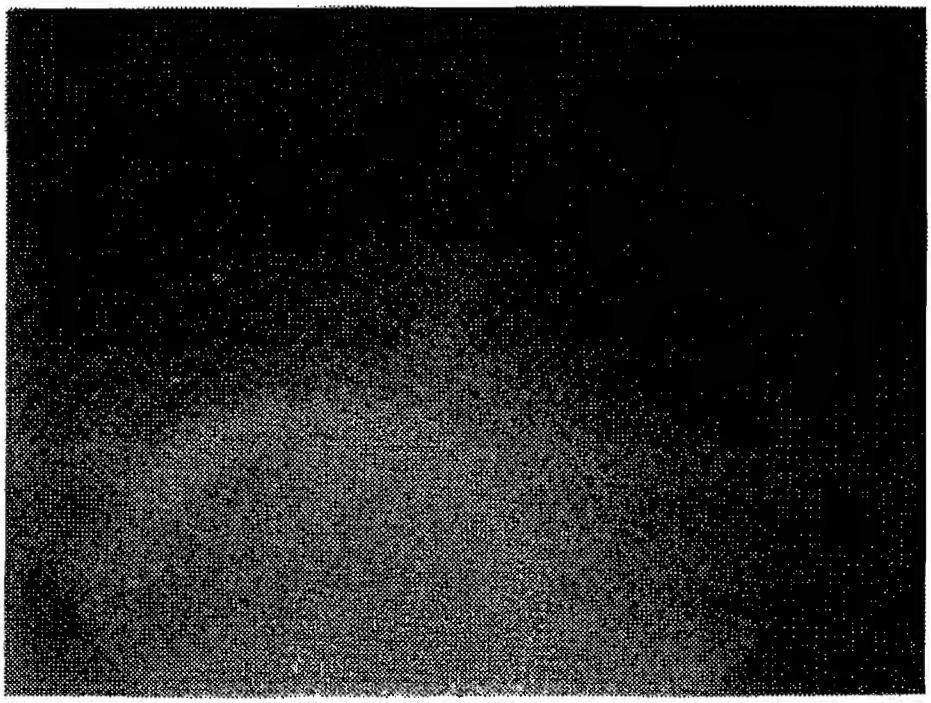

FIG. 5b

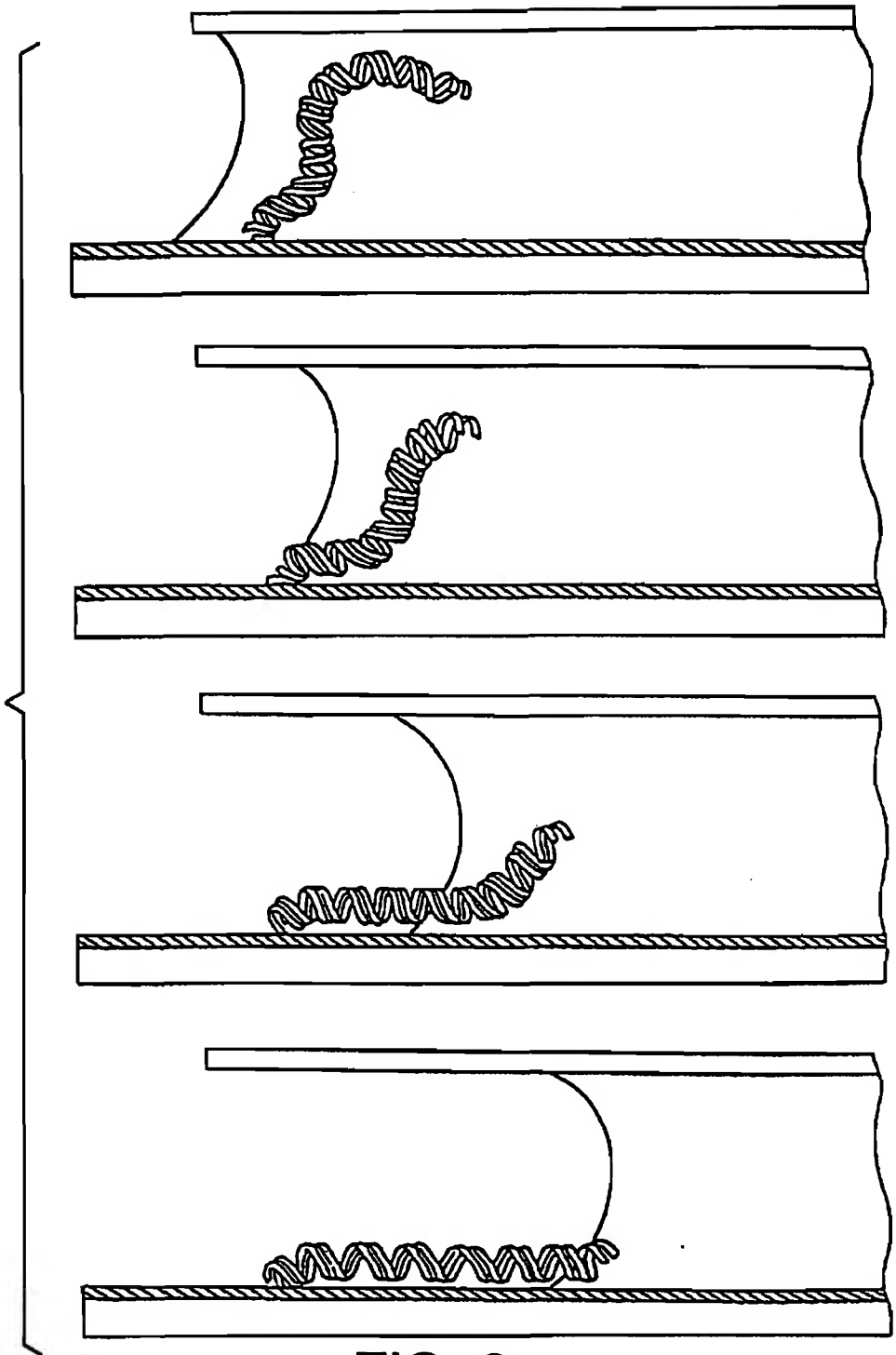

FIG. 6

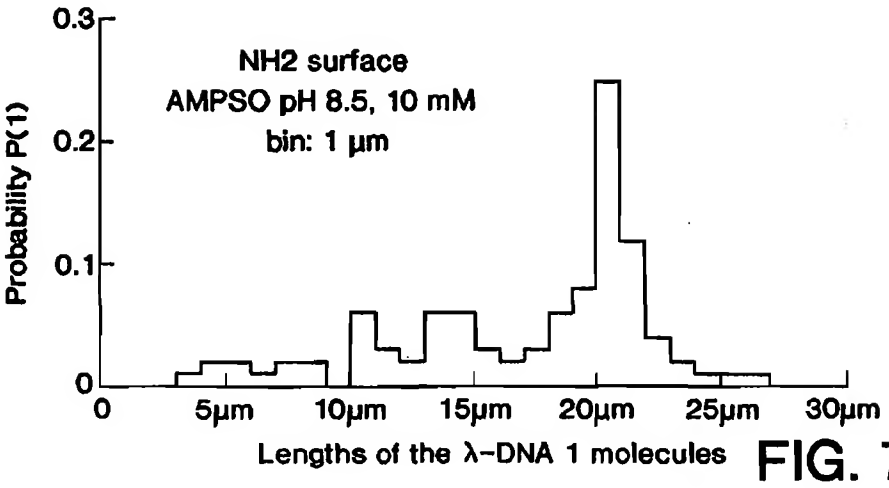

FIG. 7a

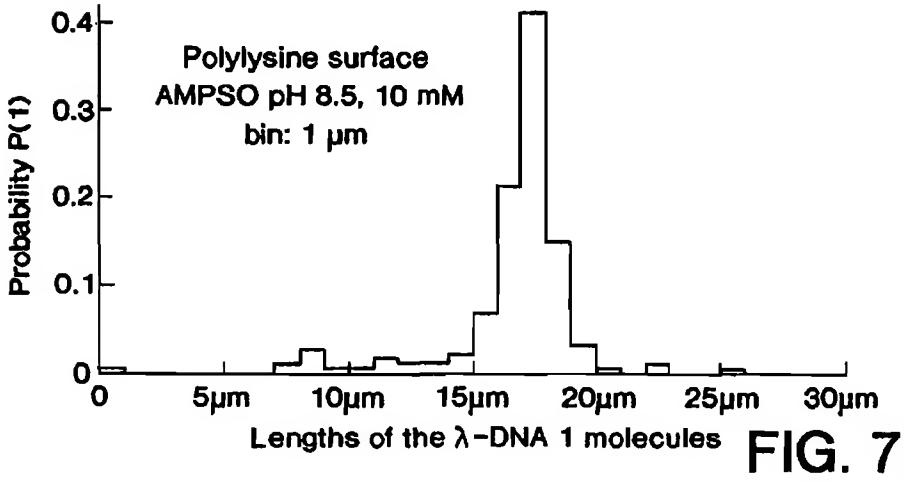

FIG. 7b

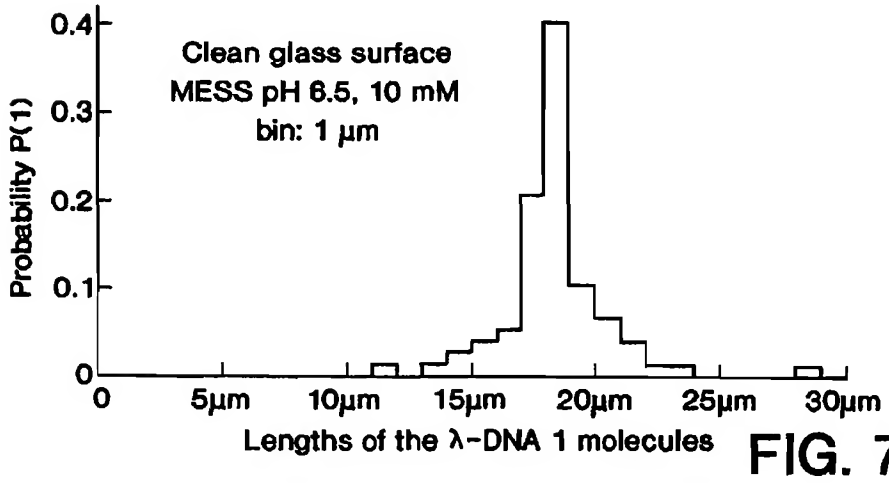

FIG. 7c

FIG. 8

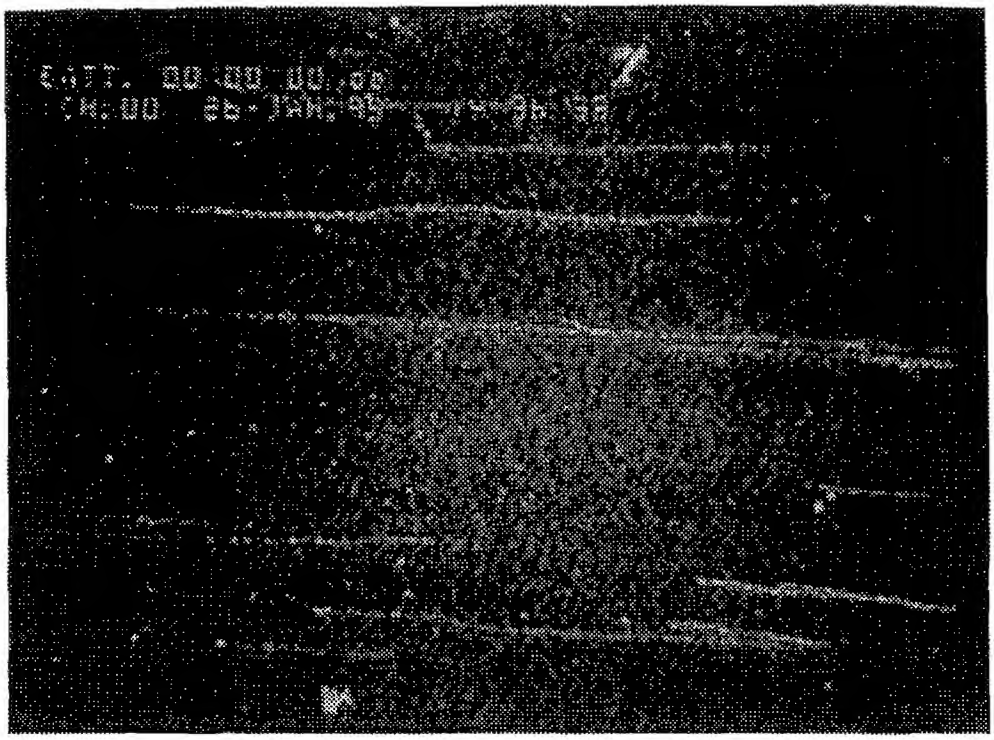

FIG. 9

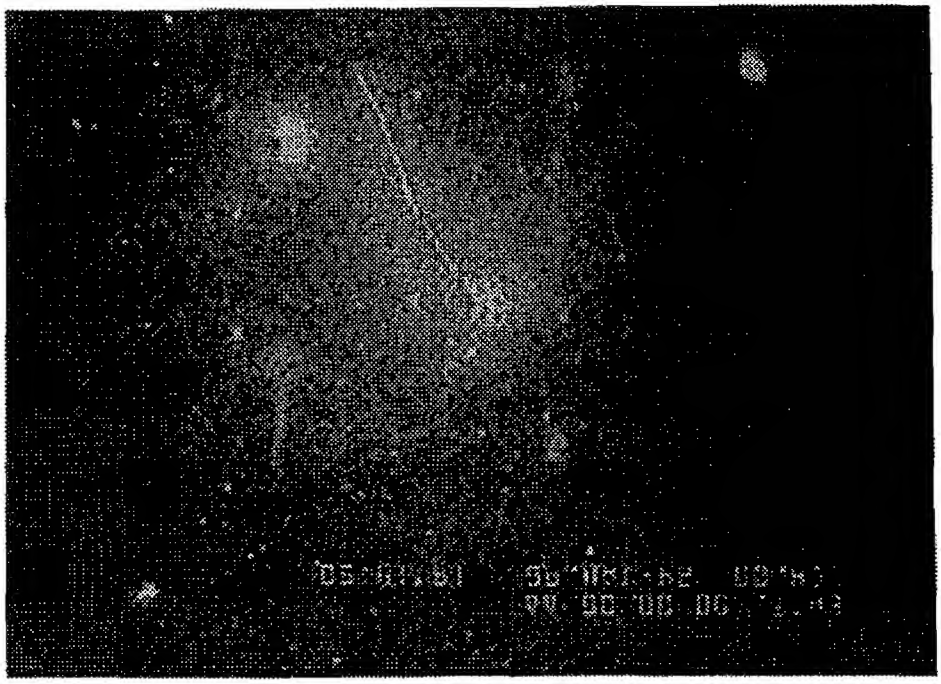

FIG. 10

MOLECULAR COMBING PROCESS FOR DETECTING MACROMOLECULES

This is a continuation of application Ser. No. 08/467,529, filed Jun. 6, 1995, U.S. Pat. No. 6,294,324, which is a continuation of application Ser. No. 08/386,485, U.S. Pat. No. 6,265,153, filed Feb. 10, 1995, now U.S. Pat. No. 6,265,153, all of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The present invention relates to a method for aligning macromolecules such as polymers or macromolecules with biological activity, especially DNA, or proteins. The present invention also relates to the application of this method in processes for detecting for measuring intramolecular distance, for separating and/or for assaying a macromolecule in a sample.

Controlling the conformation of macromolecules represents a major industrial challenge, for example in the manufacture of sensors or of controlled molecular assemblies, or alternatively in problems of detection and analysis. It may be useful to have an elongated molecular conformation. By way of example, in the case where polymers are grafted on a substrate, it has been proposed to extend them by the action of an electric field, a flow or with the aid of optical tweezers. In particular, in biology, the alignment of DNA—by electrophoresis (Zimmermann and Cox Nucl. Acid Res. 22, p 492, 1994), free flow (Parra and Windle, Nature Genetics, 5, p 17, 1993 and WO 93/22463) or in a gel (Schwartz et al. Science 262, p 110, 1993 and USP 33531) or with the aid of optical tweezers (Perkins et al., Science 264 p 819, 1994 and also U.S. Pat. No. 5,079,169)—opens numerous possibilities in mapping, or in the detection of pathogens.

These methods only allow in general an imperfect alignment, or alternatively a transient alignment—that is to say that relaxation of the molecule occurs once the stress disappears. In the case of optical tweezers, the method is expensive, is limited to only one molecule at a time, and is difficult to carry out by non-qualified staff.

A special technique for aligning DNA by flow after cell lysis, followed by drying, has been proposed (I. Parra and B. Windle and WO 93/22463). The alignment obtained is very imperfect and nonhomogeneous and numerous nonaligned masses are observed.

SUMMARY OF THE INVENTION

The subject of the present invention is a novel and simple method for aligning macromolecules on the surface S of a support, characterized in that the triple line S/A/B (meniscus) resulting from the contact between a solvent A and the surface S and a medium B is caused to move on the said surface S, the said macromolecules having a part, especially an end, anchored on the surface S, the other part, especially the other end, being in solution in the solvent A.

It has been observed according to the present invention that the mere passage of a meniscus over molecules of which one part is anchored on a substrate, the remainder of the molecule existing freely in solution makes it possible to align them uniformly, perpendicularly to the moving meniscus, leaving them adsorbed on the surface behind the meniscus. This phenomenon is called "molecular combing" here.

BRIEF DESCRIPTION OF THE DRAWINGS

The following description is made with reference to the accompanying Figures in which:

FIG. 1 schematically represents the detection of a pathogen in a fluorescent DNA molecule by hybridization with an anchor molecule.

FIG. 2 schematically represents genetic mapping by extension of DNA and the use of a marker DNA.

FIG. 3 schematically represents the detection of an immunological reaction (ELISA) by means of a "flag" molecule: a fluorescent DNA used as reaction marker.

FIG. 4 is a fluorescence micrograph showing the extension of A phage DNA by the progression of a meniscus. DNA molecules in solution stretched by the evaporation flow parallel to the meniscus and can be seen on the left. DNA molecules in the open air after being stretched perpendicularly to the meniscus can be seen on the right.

FIGS. 5(*a*) and 5(*b*) are fluorescence micrographs showing, respectively, a DNA labeled with digoxigenin (DIG) on a surface coated with anti-DIG and stretched by a meniscus, and, as control, an unlabeled DNA on an anti-DIG surface. The very high specificity of the surface and the absence of nonspecific anchoring will be noted.

FIG. 6 is a schematic representation of the spread of DNA by passage of a meniscus. DNA in solution is anchored on a treated surface. The DNA solution is covered with an untreated round cover slip.

FIG. 7 contains histograms of the length of combed A DNA molecules on glass surfaces:

a) coated with silane molecules ending with an amine group,

b) coated with polylysine, and

c) cleaned in a hydrogen peroxide/sulfuric acid mixture.

FIG. 8 represents combed DNA molecules on glass surfaces coated with polylysine. It can be noted that the molecules attached by their two ends form loops.

FIG. 9 represents YACs combed by removal of a treated cover slip in a solution of these molecules.

FIG. 10 shows that the identification of the presence and the size of a cosmid on a YAC by in situ hybridization.

More specifically, the stretching of the free part of the molecule is achieved by the passage of the triple line S/A/B constituting the meniscus between the surface S, the solvent A and a medium B which may be a gas (in general air) or another solvent.

In a specific embodiment, the meniscus is a water/air meniscus, that is to say that the solvent A is an aqueous solution and the medium B is air.

Furthermore, it is possible to extend the air/water meniscus used here in order to stretch the molecule to other systems such as oil/water or water/surfactant/air, in particular.

The movement of the meniscus can be achieved by any means of relative movement of the fluids A and B relative to the surface S. In one embodiment, the surface S can be removed from the solvent A or conversely, the solvent A can be removed from the surface S.

In particular, the meniscus can be moved by mechanical means, especially by pneumatic means by aspirating or blowing a gas, or especially by hydraulic means by pushing or aspirating the solvent A or the medium B.

Thus, the movement of the meniscus can be achieved by gradual evaporation of the solvent A.

When the movement of the meniscus is achieved mechanically, it can be achieved either by translation of the interface A/B, or by translation of the surface S.

In a specific embodiment, the solvent is placed between two supports of which at least one corresponds to the said support of surface S and the meniscus is moved for example by evaporation.

By "support", there is understood here any substrate whose cohesion is sufficient to withstand the passage of the meniscus.

The support may consist, at least at the surface, of an organic or inorganic polymer, a metal especially gold, a metal oxide or sulfide, a semiconductor element or an oxide of a semiconductor element, such as silicon oxide or a combination thereof, such as glass or a ceramic.

There may be mentioned more particularly glass, surface oxidized silicon, graphite, mica and molybdenum sulfide.

As "support", there may be used a single support such as a slide, beads, especially polymer beads, but also any form such as a bar, a fiber or a structured support, and also particles, whether it be powders, especially silica powders, which can moreover be made magnetic, fluorescent or colored as known in the various assay technologies.

The support is advantageously in the form of cover slips. Preferably, the support has little or no fluorescence.

Macromolecules, such as ordinary polymers, or biological polymers such as DNA, RNA or proteins, can be anchored by ordinary methods on a support.

The macromolecule to be aligned can be chosen from biological macromolecules such as proteins, especially antibodies, antigens, ligands or their receptors, nucleic acids, DNA, RNA or PNA, lipids, polysaccharides or derivatives thereof.

It was observed according to the present invention, that the stretching force acts locally within the immediate vicinity of the meniscus. It is independent of the length of the molecule, of the number of molecules anchored, and within a wide range, of the speed of the meniscus. These characteristics are particularly important for aligning the molecules homogeneously and reproducibly.

It is possible, according to the present invention, to add surfactant elements into the solvent A and/or the medium B, which modify the properties of the interfaces. According to the present invention, the stretching can indeed be controlled by the addition of surfactants, or by an adequate surface treatment.

Too high a surface-macromolecule attraction (for example an excessively high level of adsorption) can interfere with the alignment of the molecules by the meniscus, these molecules remaining adsorbed at the surface in a state which is not necessarily stretched. Preferably, the surface exhibits a low rate of adsorption of the said macromolecule, such that only the anchored molecules will be aligned, the others being carried by the meniscus.

However, it is possible to vary the differences in adsorption between a part of the macromolecule, especially its ends, and its other parts (in particular for long molecules such as DNA or collagen) in order to anchor, by adsorption, the molecules by a part, especially their end(s) alone, the remainder of the molecule existing freely in solution, on a wide variety of surfaces and align them by the passage of the meniscus as described above.

The adsorption of a macromolecule onto a surface can be easily controlled by means of the pH or of the ionic content of the medium or of an electric voltage applied over the surface. The surface charges and the electrostatic (repulsive or attractive) interactions between the surface and the molecule are thus changed, thereby making it possible to pass from a state of complete adsorption of the molecule onto the surface to a total absence of adsorption. Between these two extreme cases, there is a range of control parameters where the adsorption occurs preferably through the end of the molecules and which will therefore be used advantageously to anchor them on the surface, and then to align them by the passage of the meniscus.

Once aligned, the molecules adhere strongly to the surface. In the case of DNA, it was possible to observe them by fluorescence several months after their alignment.

The present invention is therefore very different from the method proposed by Parra and Windle, because according to the present invention, the molecules are anchored on the surface and then uniformly aligned by the passage of the meniscus, whereas in the Parra and Windle method, a hydrodynamic flow is used to stretch the molecules nonhomogeneously, which molecules will become nonspecifically adsorbed onto the surface.

Other techniques can also result in the stretching and the alignment of molecules. Thus, a dynamic orientation of molecules in solution, anchored at one end, can be obtained by electrophoresis or by a hydraulic flow. However, the results observed show that these techniques are much less efficient than the use of the meniscus.

By "anchoring" of the macromolecule on the surface, there should be understood an attachment resulting from a chemical reactivity both through a covalent linkage and a noncovalent linkage such as a linkage resulting from physicochemical interactions, such as adsorption, as described above.

This anchorage of the macromolecule can be achieved directly on (or with) the surface, or indirectly, that is to say via a linkage such as another molecule, especially another molecule with biological activity. When the anchorage is achieved indirectly, the macromolecule can be grafted chemically on the said linkage, or can interact physicochemically with the said linkage, in particular when the said intermediate linkage is a molecule with biological activity recognizing and interacting with the said macromolecule.

In one embodiment, the macromolecule and the said linkage are both molecules with biological activity which interact, such as an antigen and an antibody respectively, complementary nucleic acids or lipids. In these cases, the noncovalent attachment of the macromolecule consists of a linkage of the type: antigen-antibody, ligand-receptor, hybridization between complementary nucleic acid fragments or hydrophobic or hydrophilic interaction between lipids.

Advantage is thus taken of the very high specificity and the very high selectivity of certain biological reactions, especially antigen-antibody reactions, DNA or RNA hybridization reactions, interprotein reactions or avidin/streptavidin/biotin type reactions, as well as reactions of ligands and their receptors.

Thus, in order to carry out the direct or indirect anchoring of the macromolecule on the surface S, it is possible to use a solid surface having certain specificities. It is in particular possible to use certain pretreated surfaces which make it possible to attach certain proteins or DNA, whether modified or otherwise.

Such surfaces are commercially available (Covalink, Costar, Estapor, Bangs, Dynal for example) in various forms having at their surface COOH, NH_2 or OH groups for example.

It is, in this case, possible to functionalize the DNA with a reactive group, for example an amine, and carry out a reaction with these surfaces. However, these methods require specific functionalization of the DNA to be attached.

A technique allowing anchorage without prior treatment of the DNA has also been described. This process consists in causing a free phosphate at the 5' end of the DNA molecule to react with a secondary amine of the surface (NH Covalink surface).

Anchoring by adsorption can be achieved by adsorption of the end of the molecule by controlling the surface charge

by means of the pH, the ionic content of the medium or the application of an electric voltage over the surface given the differences in adsorption between the ends of the molecule and its middle part. According to the present invention, nonfunctionalized DNA molecules were thus anchored, by way of example, on surfaces coated with molecules ending with a vinyl or amine group such as polylysine molecules, or various surfaces such as glass, coated with silane type molecules ending with vinyl or amine groups or alternatively glass cover slips previously cleaned in an acid bath. In this latter case, the surface of the glass indeed has SiOH groups.

In all these cases, the pH range where the DNA is anchored is chosen to be between a state of complete adsorption and an absence of adsorption, the latter being situated at a more basic pH. It is understood that this technique is very general and can be extended by persons skilled in the art to numerous types of surfaces.

It is also possible to functionalize the DNA with a first reactive group or a protein P_0 in order to cause it to react with a surface coated with a second reactive group or with a protein P_1, which are capable of reacting specifically with each other respectively, that is to say for example P_1 with P_0. The P_0/P_1 pair may be a pair of the type: biotin/streptavidin (Zimmerrann and Cox) or digoxigenin/antibody directed against digoxigenin (anti-DIG) for example (Smith et al., Science 258, 1122 (1992)).

Preferably, the anchoring surfaces will have a low fluorescence level so as not to interfere with the detection of the molecules after their alignment, in particular if the detection is done by fluorescence.

According to the present invention, a solid support having, under the reaction. conditions, a surface having an affinity for only part of the macromolecule, the rest of the macromolecule remaining freely in solution, is preferably used.

In one embodiment, a solid support is used which has at the surface at least one layer of an organic compound having, outside the layer, an exposed group having an affinity for a type of molecule with biological activity which may be the said molecule itself or a molecule recognizing and/or interacting with it.

The support can therefore have a surface coated with a reactive group or with a molecule with biological activity.

By "affinity", there should be understood here both a chemical reactivity and an adsorption of any type, this under optional conditions of attachment of the molecules onto the exposed group, modified or otherwise.

In one embodiment, the surface is essentially compact, that is to say that it limits access by the macromolecule with biological activity to the inner layers and/or to the support, this in order to minimize nonspecific interactions.

It is also possible to use surfaces coated with a reactive exposed group (for example NH_2, COOH, OH, CHO) or with a macromolecule with biological activity (for example: proteins, such as streptavidin or antibodies, nucleic acids such as oligonucleotides, lipids, poly-saccharides and derivatives thereof) which is capable of attaching an optionally modified part of the molecule.

Thus, surfaces coated with streptavidin or with an antibody according to known processes ("Chemistry of Protein Conjugation and Cross-linking", S. C. Wong, CRC Press (1991)) are capable of attaching a macromolecule having, at a specific site, a biotin or an antigen.

Likewise, surfaces treated so as to have single-stranded oligonucleotides can serve in order to anchor on them DNAs/RNAs having a complementary sequence.

Among the surfaces having an exposed reactive group, there may be mentioned those on which the exposed group is a —COOH, —CHO, NH_2, —OH group, or a vinyl group containing a double bond —CH=CH_2 which is used as it is or which can be activated so as to give especially —CHO, —COOH, —NH_2 OR OH groups.

The supports with highly specific surfaces according to the present invention can be obtained using various processes. There may be mentioned by way of example:

(A) a layer of carbon-containing, optionally branched, polymer at least 1 nm thick, having reactive groups as defined below and

(B) surfaces obtained by depositing or anchoring on a solid support one or more molecular layers; the latter can be obtained by forming successive layers attached through noncovalent linkages, as non-limiting example, Langmuir-Blodgett films, or by molecular self assembly, this allowing the formation of a layer attached by covalent linkage.

In the first case, the surface can be obtained by polymerization of at least one monomer generating at the surface of the polymer the said exposed group, or alternatively by partial depolymerization of the surface of a polymer to generate the said exposed group, or alternatively by deposition of polymer.

In this process, the polymer formed has vinyl linkages such as a polyene derivative, especially surfaces of the synthetic rubber type, such as polybutadiene, polyisoprene or natural rubber.

In the second case, the highly specific surface contains:

on a support, a substantially monomolecular layer of an organic compound of elongated structure having at least:

an attachment group having an affinity for the support, and

an exposed group having no or little affinity for the said support and the said attachment group under attachment conditions, but optionally having, after chemical modification following the attachment, an affinity for one type of biological molecule.

The attachment can first of all be of the noncovalent type, especially of the hydrophilic/hydrophilic and hydrophobic/hydrophobic type, as in Langmuir-Blodgett films (K. B. Blodgett, J. Am. Chem. Soc. 57, 1007 (1935).

In this case, the exposed group or the attachment group will be either hydrophilic or hydrophobic, especially alkyl or haloalkyl groups such as CH_3, CF_3, CHF_3, CH_2F, the other group being hydrophilic.

The attachment can also be of the covalent type, the attachment group will, in this case, react chemically with the support.

Certain surfaces of similar structure have already been mentioned in the electronic field, especially when the attachments are covalent, L. Netzer and J. Sagiv, J. Am. Chem. Soc. 105, 674 (1983) and U.S. Pat. No. 4,539,061.

Among the attachment groups, there must be mentioned more particularly the groups of the metal alkoxide or semiconductor type, for example silane, especially chlorosilane, silanol, methoxy- and ethoxysilane, silazane, as well as phosphate, hydroxyl, hydrazide, hydrazine, amine, amide, diazonium, pyridine, sulfate, sulfonic, carboxylic, boronic, halogen, acid halide, aldehyde groups.

Most particularly, as attachment group, groups capable of cross-reacting with an adjacent equivalent group, to give cross-linkages will be preferably used; for example they will be derivatives of the metal alkoxide or semiconductor type, for example silane, especially dichlorosilane,

trichlorosilane, dimethoxysilane or diethoxysilane and trimethoxy- or triethoxysilane.

The choice of the attachment group will obviously depend on the nature of the support; the silane-type groups are quite suitable for covalent attachment on glass and silica.

As regards the exposed groups, irrespective of the surface, they will be preferably chosen from ethylenic groups, acetylenic groups or aromatic radicals, primary, tertiary or secondary amines, esters, nitriles, aldehydes, halogens. But they may be most particularly the vinyl group; indeed, the latter can be either chemically modified after attachment to give, for example, a carboxylic group or derivatives of carboxylic groups such as alcohol groups, aldehyde groups, ketone groups, acidic groups, primary, secondary or tertiary amines, or to lead to a pH-dependent direct anchoring of the biological macromolecules such as nucleic acids and proteins, without chemical modification of the surface or of the macromolecules.

Preferably, the chains connecting the exposed group to the attachment group are chains carrying at least 1 carbon atom, preferably more than 6 and in general from 3 to 30 carbon atoms.

As regards the support itself, the use of glass, surface-oxidized silicon, a polymer or gold with or without pretreatment of the surface, is generally preferred.

In the case of glass or silica, there can be used advantageously the known techniques for surface function alization using silane derivatives, for example: Si—OH+Cl_3—Si—R—CH=CH_2 gives Si—O—Si—R—CH=CH_2, R consisting for example of $(CH_2)_4$. Such a reaction is known in literature, with the use of ultrapure solvents. The reaction leads to a layer of molecules having their C=C end at the surface exposed to the outside.

In the case of gold, this being optionally in the form of a thin layer on a substrate, the known techniques for surface functionalization use thiol derivatives, for example: Au+HS—R—CH=CH_2 gives Au—S—R—CH=CH_2, R consisting for example of $(CH_2)_4$. Such a reaction is described in liquid medium and leads, like the preceding trichloro-silane-silica reaction, to a layer of molecules having their C=C end at the surface exposed to the outside.

Of course the term "support" encompasses both a single surface such as a slide, but also particles, either silica powder or polymer beads, and also ordinary forms such as a bar, a fiber or a structured support, which can moreover be made magnetic, fluorescent or colored, as is known in various assay technologies.

Preferably, the support will be chosen so as to have no or little fluorescence when the detection will be carried out by fluorescence.

The surfaces obtained according to methods (A) or (B) above have:

(i) a very low level of intrinsic fluorescence, when necessary, a fluorescence background noise (with a typical surface area of 100x100 μm) smaller than the fluorescence signal of a single molecule to be detected;

(ii) the possibility of detecting isolated molecules with an S/N ratio independent of the number of molecules, which is possible by virtue of various techniques with a high S/N ratio which are described below and which are based on the identification of the presence of a macroscopic marker having a weak nonspecific interaction with the surface.

The surfaces thus obtained are preferably coated with a macromolecule with biological activity chosen from:

proteins,

nucleic acids

lipids

polysaccharides and derivatives thereof.

Among the proteins, there should be mentioned antigens and antibodies, ligands, receptors, but also products of the avidin or streptavidin type, as well as derivatives of these compounds.

Among the RNAs and DNAs, there should also be mentioned the α, β derivatives as well as the thio derivatives and mixed compounds such as PNAs.

It is also possible to attach mixed compounds such as glycopeptides and lipopolysaccharides for example, or alternatively other elements such as viruses, cells in particular, or chemical compounds such as biotin.

The attachment of the biological macromolecules may be covalent or noncovalent, for example by adsorption, hydrogen bonds, hydrophobic, ionic interactions for example, in which case cross-linking can be advantageously carried out in the molecules grafted by known methods ("Chemistry of *Protein Conjugation and Cross-linking*", S. C. Wong, CRC Press (1991)) and this in order to enhance their cohesion.

As mentioned above, it is possible to have an exposed group which allows direct reaction with molecules with biological activity, but it is also possible to envisage that the exposed group is treated, after attachment, so as to be converted, as indicated above, to a hydroxyl, amine, alcohol, aldehyde, ketone, COOH radical or a derivative of these groups before attachment of the biological molecule.

When such groups were exposed, techniques for attachment of proteins and/or of DNA for example are known, they are indeed reactions implemented for surfaces which are already used for biological analysis, especially for Costar surfaces, Nunc surfaces or microbeads such as Estapor, Bang and Dynal for example, on which molecules of biological interest, DNA, RNA, PNA, proteins or antibodies for example, are anchored.

In the case where the exposed group is a —CH=CH_2 radical which is called hereinafter "surface C=C" or "surface with ethylenic bond", no document exists which mentions direct anchoring, in particular of DNA or of proteins.

Within the framework of the present invention, it has been demonstrated that these surfaces have a highly pH-dependent reactivity. This characteristic makes it possible to anchor the nucleic acids or the proteins using pH regions and often with a reaction rate which can be controlled by the pH.

The anchoring of DNA can be carried out by its end onto a surface having groups with ethylenic double bonds, by bringing the DNA into contact with the surface at a pH of less than 8.

In particular, the reaction is carried out at a pH of between 5 and 6, and is then stopped at pH 8.

Thus, for DNA at pH 5.5, the anchoring reaction is complete in one hour (if it is not limited by diffusion) and occurs via the ends. At pH 8 on the other hand, the attachment is very low (reaction rate of 5 to 6 orders of magnitude smaller). This pH dependent attachment effect specific for the ends, is an improvement compared with the other surfaces which require functionalization of the DNA (biotin, DIG, NHS, and: the like) or specific reagents (carbodiimide, dimethyl pimelidate) which form a peptide or phosphorimide linkage between —NH, and —COOH or —POOH.

It is also possible to carry out the anchoring of DNA by adsorption of its ends alone onto a surface coated with polylysine or a silane group ending with an amine group.

In order to carry out the anchoring of the DNA by its end on a surface coated with an amine group, the DNA is brought into contact with the surface at a pH of between 8 and 10.

Likewise, it is possible to carry out the anchoring of DNA by its end onto a glass surface treated beforehand in an acid bath, by bringing the DNA into contact with the said surface at a pH of between 5 and 8.

It goes without saying that the present invention involves, in the same spirit, the optionally pH-dependent attachment of all macromolecules of biological interest.

Likewise, these surfaces can anchor proteins directly (protein A, anti-DIG, antibodies, streptavidin and the like). It has been observed that (i) the activity of the molecule can be preserved and (ii) that the reactivity of the prepared surface (initially C═C) is completely overshadowed in favor of the sole reactivity of the molecule of interest. It is therefore possible, starting with a relatively high initial reactivity, to pass to a surface having a very highly specific reactivity, for example that of specific sites on a protein.

By anchoring a specific antibody on the surface (for example anti-DIG), a surface is created whose reactivity is limited to the antigen (for example the DIG group). This indicates that the initial chemical groups are all occulted by the antibodies grafted.

It is also possible to graft onto the reactive (chemically or biochemically) surfaces other molecules with biological activity, especially viruses or other components: membranes, membrane receptors, polysaccharides, PNA, in particular.

It is also possible to attach the product of a reaction of biological interest (for example PCR) onto the prepared surfaces.

The process according to the present invention allows the detection and/or the quantification of biological molecules, but also the measurement of intramolecular distance, the separation of certain biological molecules, especially a sample using antigen/antibody and/or DNA/RNA coupling techniques.

In particular, the subject of the present invention is a process for detecting a macromolecule, consisting of a DNA sequence or a protein in a sample, according to the present invention, characterized in that:

- the sample corresponding to solvent A, in which the said macromolecule is in solution, is brought into contact with the surface of the support under conditions for forming a DNA/DNA, DNA/RNA hybrid or for forming the protein/protein reaction product,
- the hybrid or a macromolecule for labeling the hybrid or the reaction product being anchored in one part, the remainder being free in solution, it is stretched by the movement of the meniscus created by the contact between the solvent and the surface in order to orientate the hybrids or the said labeling macromolecules and the measurement or the observation of the hybrids or of the said labeling macromolecules thus orientated is carried out.

Advantageously, the attached DNA and the DNA of the sample are colored differently and after stretching, the position of the complementary sequence relative to the end of the sample DNA is measured.

Appropriately, the ELISA or FISH detection methods can be used.

The DNA sample may be the product or the substrate of a DNA enzymatic amplification such as PCR, that is to say that the amplification of the DNA can be carried out once it has been anchored and aligned according to the process of the invention or before its anchoring or its alignment.

The passage of the meniscus, by stretching the molecules linearly, in the form of rods, renders them more easily detectable if they are labeled. Moreover, these elongated molecules are stable to the open air and can be observed even after several months, without showing apparent degradation.

During a rehydration, the DNA molecules can remain adsorbed and elongated. Furthermore, it is possible to carry out a hybridization on the elongated molecule.

Furthermore, exhibiting a signal which is correlated and of uniform orientation by virtue of their stretching, these molecules are distinct from the surrounding noise. It is therefore easy to ignore the dusts, the inhomogeneities, which have no special spatial correlation. The alignment is also important because in solution, the molecules in the form of a random cole fluctuate thermally, thereby causing very high variations in their fluorescence signal gathered preferably with a small depth of field and limits their observation. The present invention therefore allows the observation of isolated molecules with a very high signal to noise (S/N) ratio.

It is remarkable that this ratio is independent of the number of molecules anchored. The S/N ratio posed by the detection of a molecule is the same as that for 10,000. Furthermore, this stretching technique makes it possible to easily discriminate between molecules of varying lengths.

It is advantageously possible to proceed to the following stages in order to further improve the S/N ratio:

- The molecule being stationary, its fluorescence signal can be integrated.
- Microscopic observation presents a reduced field (typically 100 μm×100 μm with a ×100 immersion lens, N.A.=1.25). For a 1 cm^2 sample, scanning can be carried out, or it is possible to envisage the use of lower magnification lenses (×10 or ×20) but with a high numerical aperture.
- The rods being always parallel, it is possible to envisage an optical spatial filtration method in order further to increase the S/N ratio.
- Other global fluorescence methods can be envisaged such as those described in European Patent Application EP 103426.
- The linearization of the molecules is observed both within the framework of a physicochemical anchoring and in the case of immunological type linkages (DIG/anti-DIG).
- Once the surface is in the open air, the DNA molecules are stable (they maintain their integrity even after several weeks) and fluorescent. This property can be advantageously used in order to defer the anchoring stage and the locating/counting stage for the molecules anchored, if this detection is done for example, but without being limited thereto, by fluorescence microscopy. Such a use is covered by the present invention.
- A double (or multi) fluorescence technique can possibly be used to improve the S/N ratio or to detect a double functionality.

The stretched molecules can be revealed by various enzymological methods on others, such as fluorescence, or the use of radioactive or nonradioactive probes. Their detection can be achieved by measuring a global signal (for example the fluorescence) or by individual observation of the molecules by optical fluorescence microscopy, electron microscopy, local probe methods (STM, AFM and the like).

Thus in general, the present invention allows the detection, separation and/or assay of a molecule in a sample, by a process characterized in that a surface capable of specifically attaching the said molecule is used, and in that the detection, separation or assay are carried out using a

reagent, fluorescent or otherwise, which detects the presence of the attached molecule.

Among the reagents, there are fluorescent reagents and nonfluorescent reagents.

The fluorescent reagents contain fluorescent molecules, advantageously chosen to be long molecules of size greater than 0.1 μm and reacting specifically, directly or indirectly, with the pretreated surfaces. For example, but with no limitation being implied, a double-stranded DNA molecule stained by means of fluorescent probes (ethidium bromide, YOYO, fluorescent nucleotides and the like) capable of anchoring directly via one or more ends on a surface optionally having a vinyl or amine type group and the like, especially by a judicious choice of the pH or of the ionic content of the medium or by application of an electric voltage over the surface.

It is also possible to use a special functionalization of the molecule (DIG, biotin and the like) in order to anchor it at one or more points on a surface having complementary sites (anti-DIG, streptavidin and the like).

Nonfluorescent reagents allowing the detection of molecules previously aligned according to the present invention may consist especially of beads or micro-particles anchored via another molecule attached specifically, directly or indirectly, to the aligned molecule and having only a weak nonspecific interaction with the surface.

For example, there may be mentioned Dynal beads coated with streptavidin permitting anchoring on biotinylated DNA aligned according to the present invention.

Depending on whether the desired molecule is detected directly by fluorescence or indirectly by means of the above reagents, the detection will be described as "direct detection" or "flag detection".

In order to limit the problems associated with too slow reaction times, the diffusion times of the reagents towards the surface can be advantageously reduced using small reaction volumes. For example, but with no limitation being implied, by carrying out the reaction in a volume of a few microliters determined by the space between two surfaces of which one is treated so as to have reactive sites and the other is inert or treated so as not to have reactive sites, under the reaction conditions.

The detection of the number of aligned molecules can be carried out on a small number of molecules (typically 1 to 1000), by a low-noise macroscopic physical test requiring neither electron microscope nor radioactivity nor necessarily PCR.

The alignment and detection processes according to the present invention are capable of being carried out by persons having only limited laboratory experience.

The specificity of certain biological reactions may be limited. Thus, within the framework of the hybridization, the hybrids may be imperfect (reactions with other sites) while having a reduced number of pairing and therefore a lower quality of binding. The present invention also covers the possible use of a stage for testing the quality of the bonds obtained. This test makes it possible to dissociate the products weakly and nonspecifically paired by adsorption, hydrophobic forces, imperfect hydrogen bonds, imperfect hybridization, in particular.

Accordingly, the invention also relates, in a detection or assay process as described above, to a process where the product of the reaction between the molecule with biological activity and the sample molecule is subjected to a stress in order to destroy the mismatches before the detection.

This process offers, in addition to the possibility of destroying the mismatched pairs, the possibility of orientating the products of the coupling, which facilitates the measurements or the observations.

It is thus possible to apply to the surfaces, after attachment of the complementary elements, a stress which may consist of the single or combined use of:

centrifugation,

gradient of magnetic field applied to the nonfluorescent reagents taken, in this case, to include magnetizable or magnetic microbeads,

stirring,

liquid flow,

meniscus passage,

electrophoresis

temperature variation, and/or temperature gradient.

The number of systems to have remained intact or to have been destroyed is then determined by the low-noise detection techniques described above.

The alignment and detection techniques described according to the present invention can be used for numerous applications among which, but with no limitation being implied:

the identification of one or more elements of DNA or RNA sequence which can be advantageously used for the diagnosis of pathogens or the physical map of a genome. In particular, the techniques described above make it possible to obtain a physical map directly on genomic DNA without the intermediate use of a cloning stage. It is understood that the combed molecule having been stretched relative to its crystallographic lengths, relative measurements are carried out. It is thus possible to measure the size of the DNA fragments and the distance between fragments, with a resolution of the order of 200 nm by optical methods or of the order of 1 nm by the use of near field methods such as AFM or STM in order to visualize and measure the distance between probes on the aligned DNA.

This naturally leads to:

1) the detection of deletions, additions or translocations of genomic sequences, in particular in the diagnosis of genetic diseases (for example Duchene's myopathy);

2) the identification of promoters of various genes by measuring the distance between the regulatory sequences and those expressed;

3) the localization of regulatory proteins by identifying their position along the DNA or the position of their target sequence;

4) the partial or complete sequencing by measuring the distance using near field microscopy (for example AFM or STM) between hybridized probes belonging to a base oligonucleotide of given length;

the enzymatic amplification in situ on aligned DNAs;

the improvement of the sensitivity of ELISA techniques with the possibility of detecting a small number (possibly less than 1000) of immunological reactions.

Thus, physical mapping can be carried out directly on a genomic DNA without the intermediate use of a cloning stage. The genomic DNA is extracted, purified, optionally cleaved with one or more restriction enzymes and then combed on surfaces according to the process of the present invention.

The position and size of the desired gene on the genomic DNA are then determined by hybridization with probes specific for the said gene, especially extracted from parts of the complementary DNA (cDNA) of the product of the said desired gene.

Similarly, by hybridizing a genomic DNA combed, then denatured with total cDNA labeled by fluorescence or any other marker allowing the hybrid to be localized, the position, size and number of exons of the gene in question are identified and its size and its genetic organization (exons, introns, regulatory sequences) are deduced therefrom.

The position of the gene having been determined as described above or being known, it is then possible to identify, by hybridization, the flanking sequences of the gene. For that, the procedure is advantageously carried out by hybridization with labeled probes, obtained for example from an oligonucloetide library, in order to identify two or more probes which hybridize on either side of the gene.

From this determination, it is then possible, by enzymatic amplification techniques, for example in situ PCR (Nuovo G. J. PCR in situ hybridization: protocols and applications, Raven Press (1992)) to amplify the fragment delimited by the flanking probes which can serve as primers for the reaction, which fragment may contain the desired gene with its regulatory regions which may be tissue- or development-specific and which can then be isolated and purified.

The procedure can also be carried out by in situ polymerization on primers extracted from the cDNA of the gene in question in order to extract DNA fragments complementary to the flanking regions of the gene as mentioned by Mortimer et al. (Yeast 5, 321, 1989). These fragments can then serve in the preparation of primers for a process of enzymatic amplification of the gene and of its flanking sequences.

The methods cited by A. Thierry and B. Dujon (Nucl. Acid Research 20 5625 (1992)) for inserting, by homologous recombination or randomly, known endonuclease-specific sites into a genomic DNA or a genomic DNA fragment, may also be used. The combing of this DNA allows the identification of the gene of interest and of the specific sites inserted, by the in situ hybridization methods described above. From this identification and preferably, if the sites of interest are regions of interest which are close to the gene, they will be used as primer for a reaction of enzymatic amplification (in situ and the like) of the gene in question and of its flanking sequences.

The amplification of the desired gene then proceeds using known enzymatic amplification techniques such as PCR on the amplified fragment as described above, using primers which can be reached by the exons constituting the cDNA, or primers corresponding to flanking sequences.

By the combing of genomic DNA and the like, it is also possible to determine, by hybridization, the presence or the absence of regulatory sequence of a specific proximal gene, from which the possible families of proteins for regulating this gene (for example: helix-loop-helix, zinc-finger, leucine-zipper) will be determined.

The specific reactions between particular DNA/RNA/PNA sequences, and another molecule (DNA, RNA, protein) can occur before or after aligning the molecules according to the present invention.

Thus, in the field of genetic diagnosis and physical mapping, the known methods of FISH (Pinkel et al., Proc. Nat. Acad. Sci. USA 83, 2934 (1986)) are advantageously used to hybridize single-stranded oligonucleotides labeled with DNA first aligned, and then denatured. The revealing of the hybrids will be carried out using known techniques (fluorescence, microbeads and the like) with a resolution in the measurement of the distances ranging from 0.2 μm (optically) to 1 nm (by near field microscopy; AFM, STM and the like).

Alternatively, it is possible to first hybridize fluorescently labelled DNAs to single-stranded DNA in solution, and then to align this construct by action of the meniscus after having converted it to double-stranded DNA and anchored it on an appropriate surface.

It is also possible to use the present invention for detecting the presence of a pathogen. By way of example, the procedure can be carried out in two different ways depending on whether the recognition reaction (hybridization, attachment of proteins) occurs before or after alignment by the meniscus.

Thus, by way of example, one or several oligonucleotide probes are anchored in one or more regions of a surface. The hydbridization of the potentially pathogenic DNA is carried out in situ under stringent conditions so as to anchor only the hybridized molecules. Their detection and quantification is carried out after alignment by the meniscus according to the present invention.

Alternatively, the potentially pathogenic DNA is first aligned, then denatured and hybridized with an oligonucleotide probe under stringent conditions. The detection of the hybrid is then carried out by known methods, especially by the FISH method, as described above.

Similarly, it is possible to detect the presence (or the absence) of a small number of molecules, such as proteins, lipids, sugars, or antigens. A minor modification of the ELISA techniques will be advantageously carried out, the usual detection method being replaced by the detection of a fluorescent molecule aligned according to the present invention and coupled to one of the reagents of the ELISA reaction.

Moreover, as mentioned by R. R. Allan et al. (U.S. Pat. No. 84 114), genetic mapping can be carried out by measuring the size of the DNA fragments. Now, the novel techniques for stretching molecules described above (stretching by meniscus) allows the length of the stretched molecules to be measured, and this on a very small sample (a few thousandths of molecules).

It is for example possible, but with no limitation being implied, to carry out the procedure in the following manner:

A DNA sample is fragmented (by means of restriction enzymes) stained with a fluorophore and then anchored on a surface. The molecules are then stretched by the meniscus and the size of the stretched fragments is determined by optical fluorescence microscopy with a resolution and a maximum size of the order of 1000 bp (0.3 μm).

For this purpose, but also if it is desired to align very long molecules ($\geqq$10 μm), known techniques will be advantageously used in order to limit the degradation of long macromolecules during their handling (by hydrodynamic shearing).

Thus, as mentioned by D. C. Schwartz, condensation of the molecules will be advantageously carried out by means of a condensing agent (for example spermine or an alcohol) during their handling. Optionally, their decon-densing will occur during contact between the solvent A and the anchoring surface S.

In order to reduce the degradation of the macromolecules during stretching by the meniscus, meniscus translation techniques will be used which minimize hydrodynamic shearing. For example, but with no limitation being implied, by very slowly removing ($\leqq$200 μ/sec) the surface S from a substantial volume ($\geqq$100 μl) of the solvent A.

The subject of the present invention is also a surface having one or more types of aligned macromolecules obtained according to the present invention. In particular, it is possible to obtain a surface or a stack of surfaces having anisotropic optical or electrical properties.

The subject of the present invention is also a process for aligning and detecting DNA in which the DNA is stretched

by an aligning process according to the invention, then denatured and hybridized with specific probes in order to determine the position or the size of one or more specific sequences.

The subject of the present invention is also a process for the physical mapping of a gene on a genomic DNA in which the DNA is aligned or detected according to a process of the invention.

In particular, the position and the size of the desired gene on the genomic DNA are determined by hybridization with probes specific for the said gene to be mapped.

A subject of the present invention is also

a kit useful for carrying out a mapping process according to the invention, consisting of total genomic DNA from a reference host,

a support having a surface permitting the anchoring and the alignment of the patient's DNA in accordance with the process of the invention

probes specific for the gene(s) to be mapped and reagents for the hybridization and the detection of the DNA.

The subject of the present invention is also a process for aligning and detecting DNA in which the DNA is stretched, then denatured and hybridized with specific probes in order to determine the presence or the absence of one or more DNA sequences in the aligned DNA.

The present invention allows the implementation of a process for the diagnosis of a pathology related to the presence or the absence of a DNA sequence specific for the pathology in which an alignment process according to the invention is used.

The subject of the present invention is also a kit useful for carrying out a diagnostic process according to the invention, characterized in that it contains a support whose surface permits the anchoring and the alignment of the patient's DNA according to a process of the invention, probes specific for the gene involved in the sought pathology and reagents for the hybridization and the detection of the DNA.

The subject of the present invention is also a kit useful for carrying out a diagnostic process according to the invention, characterized in that it contains a support whose surface has probes specific for the gene involved in a pathology, in particular optionally labeled pathogenic DNA, which are aligned according to the process of the present invention and optionally denatured; the reagents for preparing and labeling the patient's DNA for its hybridization (for example photobiotin, nick translation or random priming kit) and reagents for the hybridization and the detection of the DNA according to the in situ hybridization techniques as described above.

It is understood that combed probes relating to different pathogens may be present on different supports or on the same support. The identification of the corresponding pathogen can be carried out after hybridization, either spatially (the different probes are spatially separated for example by photochemical anchoring before their combing) or by a difference in the fluorescence spectrum of the different hybrids, resulting from a prior differential labeling of the probes.

Finally, the subject of the present invention is a process for preparing a gene in which the position of the said gene on the genomic DNA aligned by the process according to the invention is identified by means of a probe specific for the said gene, the sequence of the said gene and optionally its flanking sequences are amplified by enzymatic amplification, in particular by in situ PCR.

The present invention therefore makes it possible to carry out a process for replacing a gene in the genome of an eukaryotic cell by targeted insertion of a foreign gene by means of a vector containing the said foreign gene prepared according to the above gene preparation process.

The targeted insertion can be carried out according to the techniques described in WO 90/11354 by transfecting eukaryotic cells with a vector containing the said foreign DNA to be inserted flanked by two genomic sequences which are contiguous to the desired site of insertion in the recipient gene. The insert DNA may contain either a coding sequence, or a regulatory sequence. The flanking sequences are chosen so as to allow, by homologous recombination, depending on the case, either the expression of the coding sequence of the insert DNA under the control of the regulatory sequences of the recipient gene, or the expression of a coding sequence of the recipient gene under the control of a regulatory sequence of the insert DNA.

The genomic genes and the cDNAs obtained using the process for localizing genes according to the invention can be inserted into expression vectors capable of being inserted into a prokaryotic, eukaryotic or viral host cell. The derived proteins, polypeptides and peptides are included in the present invention.

In the "diagnostic" mode, the probes (the "anchors") possess a reactive group (DIG, biotin and the like) capable of anchoring specifically on a surface according to the present invention (having for example as anchoring site an anti-DIG antibody or streptavidin). The detection of the anchoring reaction can be carried out directly by detection of the fluorescence of the DNA molecule stained by fluorescent molecules (ethidium bromide, YOYO, fluorescent nucleotides) (FIG. 1). It can also be carried out indirectly by detection of a "flag molecule": a reagent capable of attaching to the DNA/RNA molecule (for example by hybridization, protein-DNA interaction and the like), but having no affinity for the anchoring sites of the probe.

In the "mapping" mode, in situ hybridization techniques (FISH) can be used. It is also possible to envisage other techniques, for example by hybridizing in solution DNA with probes having fluorescent reagents according to the present invention. The detection of the position of the probes is carried out after aligning the molecule according to the prevent invention.

EXAMPLE 1

Materials and Methods

The λ DNA and the monoclonal antibody (anti-DIG) are obtained from Boehringer-Mannheim. The trichlorosilanes are obtained from Roth-Sochiel. The fluorescent nucleic probes (YOYO1, YOYO3 and POPO1) are obtained from Molecular Probes. The ultraclean glass cover slips are obtained from Erie Scientific ((ESCO) cover slips). The magnetic particles are obtained from Dynal. The microscope is a Diaphot inverted microscope from NIKKON, equipped with a Xenon lamp for epifluorescence and a Hamamatsu intensified CCD camera for the visualization.

Surface Treatment

Glass cover slips are cleaned for one hour by UV irradiation under an oxygen atmosphere (by formation of ozone). They are then immediately placed in a desiscator previously purged of traces of water by an argon stream. A volume of about 100 to 500 μl of the appropriate trichlorosilane ($H_2C{=}CH{-}(CH_2)_N{-}SiCl_3$)is introduced into the desiccator, from which the surfaces are removed after about 12 hours (n=6) or 1 hour (n=1). upon taking out, the surfaces are clean and nonwetting.

The functional groups of these double bond surfaces ($H_2C{=}CH{-}$) can be converted to carboxyl groups (—COOH) by soaking the treated cover slips, as described

above, for about ten minutes in a solution of 25 mg $KMnO_4$, 750 mg $NaIO_4$ in 1 μl of water, then by rinsing them three times in ultrapure water.

The cover slips thus functionalized can react with proteins. A volume of 300 μl of an aqueous solution (20 μg/ml) of proteins (protein A, streptavidin and the like) is deposited on a cover slip functionalized with a (H_2C=CH—) group. This cover slip is incubated for about two hours at room temperature, then rinsed three times in ultrapure water. The surfaces thus treated are clean and wetting. The surfaces treated with protein A can then react with an antibody, for example an anti-DIG antibody, by incubating in a solution of 20 μg/ml of antibody.

Moreover, on the surfaces having carboxyl groups, it is possible to graft oligonucleotides having an amine end (—NH_2), 200 μl of a solution of MES (50 mM, pH 5.5), carbodiimide (1 mg/ml) and 5 μl of amino-oligo-nucleotide (10 pmol/140 μl) are deposited on a carboxylated surface and incubated for about 8 hours at room temperature. The cover slip is finally rinsed three times in NaOH (0.4 M) and then four times in ultrapure water. The cover slips thus prepared can hybridize DNAs complementary to the anchored oligonucleotide.

Anchorina of Native DNA on a Double Bond Surface

A drop of 2 μl of a fluorescence-labeled λ DNA (YOYO1, POPO1 or YOYO3, but with no specific end labelling) of varying concentration and in different buffers (total number of molecules<10^7) is deposited on a pretreated cover slip (C=C) and covered with an untreated glass cover slip (diameter 18 mm). The preparation is incubated for about 1 hour at room temperature in an atmosphere saturated with water vapor. In a 0.05 M MES buffer (pH=5.5), a virtually general anchoring of the DNA molecules is observed. In contrast, in a 0.01 M Tris buffer (pH=8), there is practically no anchored molecule (ratio<10^6). This dependence can make it possible to control the activation/deactivation of surfaces (with respect to DNA) via the pH.

The action of the meniscus on the molecule is limited to its immediate vicinity. The part of the molecule in solution in front of the meniscus fluctuates freely and the part left stuck on the surface behind the meniscus is insensitive to a change in the direction of the meniscus. The extension rate of the molecule is therefore uniform and independent of its size.

Alignment and Detection of the Anchoring by the Action of the Meniscus

By transferring the preceding preparation to a dry atmosphere, the solution, upon evaporating, will stretch the DNA molecules anchored on the surface, perpendicularly to the meniscus. The capillary force on the DNA molecule (a few tens of picoNewtons) is indeed sufficient to completely stretch the molecule (greater than the entropic elasticity forces), but too weak to break the bond between the end of the molecule and the treated surface. The DNA having been fluorescence labeled, the stretched molecules (total length about 22 μm) can be individually and easily observed. The anchoring between the surface and the DNA being limited to the ends, it is possible to stretch either DNA of λ phage, of YAC or of *E. coli* (total length greater than 400 μm). This DNA preparation, stretched, fluorescent and in the open air, is stable for several days and can be observed in a nondestructive manner, by epifluorescence (Nikkon Diaphot inverted microscope with a ×100 lens, O.N.: 1.25).

Specific Anchoring and Detection

By treating the surfaces as described above with a specific monoclonal antibody, it is possible to control their specificity very precisely. Thus, the specificity of anti-DIG treated surfaces was tested in relation to λ DNA hybridized with an oligonucleotide complementary to one of the Cos ends and possessing a digoxigenin group (DIG) and in relation to nonhybridized DNA. In the first case, a virtually general extension of the anchored molecules, by the action of the meniscus, was observed. In the second case, only a few anchored DNA molecules (<10) were observed in the whole sample. It is therefore estimated that the specificity of the method according to the invention is greater than 10^6.

λ DNAs were also hybridized with oligonucleotides complementary to one of the COS ends and attached to carboxylated surfaces, as described above. The hybridization conditions (pure water at 40° C.) were not stringent, because under stringent conditions (high salinity) the fluorescence of the YOYO1 probes disappears and the hybridized DNAs cannot be seen. It was also possible to align the DNAs thus hybridized by passage of the meniscus.

Sensitivity of the Detection

In order to determine the sensitivity of the detection method by extension of the meniscus, 2.5 μl drops of a solution of λ DNA in 0.05 M MES (pH=5.5) containing a total of 10^5, 10^4 and 1000 molecules, were deposited on double bond surfaces. The anchoring and the alignment are carried out as described above. The cover slips are then observed by epifluorescence microscopy to determine the density of combed molecules. The latter indeed corresponds to that estimated: about 4–6 DNA molecules per field of vision (100 μm×100 μm) for a total of 10^5 DNA molecules. For the lowest concentration, it was possible to observe about ten molecules extended by the action of the meniscus. This number is essentially limited by the large number of fields of vision required to cover the whole sample (about 25,000), which makes a manual search difficult, but it can be advantageously carried out automatically or also with a weaker lens, but with a larger field. In conclusion, the sensitivity of the method according to the invention allows detection and individual counting of less than 1000 DNA molecules.

Detendence of the Stretching on the Surface Treatment

The histogram of the lengths of A DNA grafted on different surfaces and then aligned by passage of the meniscus shows a well defined peak but which is different for the different surfaces. Thus, on surfaces coated with a silane which end with a vinyl group, the DNA is stretched up to about 22 μm (see above) for surfaces silanized with an amine group (—NH_2), the histogram has a peak at 21 μm (FIG. 7(*a*)) and on clean glass at about 18.5 μm (FIG. 7(*c*)).

The stretching therefore depends on the surface treatment.

EXAMPLE 2

Combing of DNA Molecules on Different Surfaces

The molecular combing of DNA on glass surfaces treated in various ways was observed. Advantage is taken of the difference in absorption between the ends of the molecule and the rest of the molecule. By adsorbing positively charged polymers onto a glass surface, adsorption of negatively charged DNA molecules is enhanced, however, when this charge is large, the DNA molecule is stuck over its entire length and the combing is impossible. However, it is possible to modify the charge of the polymers adsorbed on the glass by modifying the pH conditions, indeed, the positive charges are carried for example by the NH_2 groups which pass to the protonated state NH_{3+} for a pH below the pK of the corresponding base. In basic pH, the charges disappear and the surface no longer attracts DNA. By finely controlling the pH, it was observed that the DNA molecules in solution passed from a state where they are completely stuck to the surface to an intermediate phase where they are

anchored only by their ends and then to a phase where the surface no longer has affinity for the DNA. In the intermediate phase, molecular combing can be carried out.

Surfaces coated with a silane ending with an NH_2 group were studied for which there is observed complete sticking at pH<8, and combing for 8.5<pH<9.5. The number of combed molecules is maximum at pH=8.5; it is divided by 2 at pH=9 and by 4 at pH=9.5. Also the relative extension on this surface which corresponds to 1.26 was determined as can be seen in histogram 2 of FIG. 7 which represents histograms of the length of the combed λ DNA molecules on glass surfaces:

a) coated with silane ending with an amine group,

b) coated with polylysine, and

c) cleaned in a hydrogen peroxide/sulfuric acid mixture.

Surfaces coated with polylysine were also examined and found to exhibit similar attachment characteristics as regards the pH: combing region at 8, 5 and exhibiting a shorter relative extension: 1.08. A typical example can be obtained in FIG. 8 which represents combed DNA molecules on glass surfaces coated with polylysine. It can be observed that the molecules attached by their two ends form loops.

Finally, the same behavior was found on glass surfaces freshly cleaned in a hydrogen peroxide/concentrated sulfuric acid mixture. These surfaces are highly wetting and become rapidly contaminated; however, a combing region was observed between 5.5<pH<7.4 whereas the region of strong adsorption is situated at pH=4.5. The relative extension of the molecules corresponds to 1.12.

EXAMPLE 3

Uniform and Directional Alignment of YAC

1 μg of YAC previously stained in its agarose plug by means of a YOYO1 fluorescent probe is heated to 68° C., agarased and then diluted in 10 ml of MES (50 mM pH 5.5). Two silanized cover slips (C═C surfaces) are incubated for ~1.5 h in this solution and then removed at about 170 μm/sec. The YAC molecules are all aligned parallel to the direction of removal of the cover slips (FIG. 9). The integrity of the molecules thus aligned is better than by evaporation after deposition between two cover slips.

Hybridization of a Cosmid with a Combed YAC

A YAC stained as previously described is anchored on a C═C surface (between two cover slips) and then aligned by the meniscus, during evaporation of the solution. The probes (cosmids) are labeled by incorporation of a biotinylated nucleotide by the random priming technique. The labeled probes (100 ng) and 5 μg of sonicated salmon sperm DNA (~500 bps) are purified by precipitation in Na-acetate and ethanol, and then denatured in formamide.

The combed YACs are denatured between two cover slips with 120 μl of denaturing solution (70% formamide, 2×SSC) on a hotplate at 80° C. for 3 minutes. The previously denatured probes (20 ng) are deposited on the cover slip in a hybridization solution (55% formamide, 2×SSC, 10% dextran sulfate) covered with a cover slip and sealed with rubber cement. The hybridization is carried out overnight at 37° C. in a humid chamber.

The detection of the hybrids is performed according to procedures known for in situ hybridizations on decondensed chromosomes (D. Pinkel et al., PNAS USA 83, 2934 (1986) and PNAS USA 85, 9138 (1988)).

Hybridized segments such as that shown in FIG. 10 are then observed by fluorescence microscopy. This example demonstrates the possibility of detecting the presence of a gene on a DNA molecule, which can be used for diagnostic purposes or for physical mapping of the genome.

What is claimed is:

1. A process for detecting a macromolecule in a sample, wherein the process comprises:

(a) providing a support having a surface, wherein the surface comprises a molecule with biological activity attached thereto and wherein the molecule with biological activity comprises biotin, a protein, a nucleic acid, a lipid, or a polysaccharide;

(b) contacting the sample with the surface to couple the macromolecule in the sample to the molecule with biological activity;

(c) passing a meniscus over the coupled product from step (b) to align the coupled product on the surface; and

(d) detecting, either directly or indirectly, the macromolecule in the coupled product.

2. The process according to claim 1, wherein the protein comprises an antibody, an antigen, a ligand, or a ligand receptor.

3. The process according to claim 1, wherein the surface comprises an organic polymer, an inorganic polymer, a metal, a metal oxide, a sulfide, or a semiconductor element.

4. The process according to claim 1, wherein the surface comprises glass, surface-oxidized silicon, gold, graphite, molybdenum sulfide, or mica.

5. The process according to claim 1, wherein the support comprises a plate, a bead, a fiber, or a particle.

6. The process according to claim 1, wherein the surface of the support comprises an exposed reactive group having an affinity for the molecule with biological activity.

7. The process according to claim 1, wherein the surface comprises a vinyl, an amine, a carboxyl, an aldehyde, or a hydroxyl group.

8. The process according to claim 6, wherein the surface of the support comprises a substantially monomolecular layer of an organic compound and wherein the substantially monomolecular layer of the organic compound comprises:

(a) an attachment group having an affinity for the support; and

(b) an exposed group having no or little affinity for the support and the attachment group under attachment conditions, but having an affinity for the molecule with biological activity.

9. A process according to claim 6, wherein the exposed reactive group is an ethylenic double bond, a vinyl group, or an amine group.

10. The process according to claim 1, wherein the protein comprises avidin or streptavidin.

11. The process according to claim 1, wherein the meniscus is passed over the coupled product by removing the surface from the sample or removing the sample from the surface.

12. The process according to claim 1, wherein the meniscus is passed over the coupled product by evaporation of the sample.

13. The process according to claim 1, further comprising subjecting the coupled product to a stress to destroy any mismatches before detecting the macromolecule.

14. A process for detecting a macromolecule in a sample, wherein the process comprises:

(a) providing a support having a surface, wherein the surface comprises a molecule with biological activity, wherein the molecule with biological activity has been aligned on the surface by passage of a meniscus over the molecule with biological activity, and wherein the molecule with biological activity comprises biotin, a protein, a nucleic acid, a lipid, or a polysaccharide;

(b) contacting the sample with the surface to couple the macromolecule in the sample to the molecule with biological activity; and

(c) detecting, either directly or indirectly, the macromolecule in the coupled product from step (b).

15. The process according to claim 14, wherein the protein comprises an antibody, an antigen, a ligand, or a ligand receptor.

16. The process according to claim 14, wherein the surface comprises an organic polymer, an inorganic polymer, a metal, a metal oxide, a sulfide, or a semiconductor element.

17. The process according to claim 14, wherein the surface comprises glass, surface-oxidized silicon, gold, graphite, molybdenum sulfide, or mica.

18. The process according to claim 14, wherein the support comprises a plate, a bead, a fiber, or a particle.

19. The process according to claim 14, wherein the surface of the support comprises an exposed reactive group having an affinity for the molecule with biological activity.

20. The process according to claim 14, wherein the surface comprises a vinyl, an amine, a carboxyl, an aldehyde, or a hydroxyl group.

21. The process according to claim 19, wherein the surface of the support comprises a substantially monomolecular layer of an organic compound and wherein the substantially monomolecular layer of the organic compound comprises:

(a) an attachment group having an affinity for the support; and

(b) an exposed group having no or little affinity for the support and the attachment group under attachment conditions, but having an affinity for the molecule with biological activity.

22. A process according to claim 19, wherein the exposed reactive group is an ethylenic double bond, a vinyl group, or an amine group.

23. The process according to claim 14, wherein the protein comprises avidin or streptavidin.

24. The process according to claim 14, further comprising subjecting the coupled product to a stress to destroy any mismatches before detecting the macromolecule.

25. A process for detecting a macromolecule in a sample, wherein the process comprises:

(a) providing a support having a surface;

(b) anchoring a molecule with biological activity to the surface, wherein the molecule with biological activity comprises biotin, a protein, a nucleic acid, a lipid, or a polysaccharide;

(c) contacting the sample with the surface to couple the macromolecule in the sample to the molecule with biological activity;

(d) passing a meniscus over the coupled product from step (c) to align the coupled product on the surface; and

(e) detecting, either directly or indirectly, the macromolecule in the coupled complex.

26. The process according to claim 25, wherein the protein comprises an antibody, an antigen, a ligand receptor.

27. The process according to claim 25, wherein the surface comprises an organic polymer, an inorganic polymer, a metal, a metal oxide, a sulfide, or a semiconductor element.

28. The process according to claim 25, wherein the surface comprises glass, surface-oxidized silicon, gold, graphite, molybdenum sulfide, or mica.

29. The process according to claim 25, wherein the support comprises a plate, a bead, a fiber, or a particle.

30. The process according to claim 25, wherein the surface of the support comprises an exposed reactive group having an affinity for the molecule with biological activity.

31. The process according to claim 25, wherein the surface comprises a vinyl, an amine, a carboxyl, an aldehyde, or a hydroxyl group.

32. The process according to claim 30, wherein the surface of the support comprises a substantially monomolecular layer of an organic compound and wherein the substantially monomolecular layer of the organic compound comprises:

(a) an attachment group having an affinity for the support; and

(b) an exposed group having no or little affinity for the support and the attachment group under attachment conditions, but having an affinity for the molecule with biological activity.

33. A process according to claim 30, wherein the exposed reactive group is an ethylenic double bond, a vinyl group, or an amine group.

34. The process according to claim 25, wherein the protein comprises avidin or streptavidin.

35. The process according to claim 25, wherein the meniscus is passed over the coupled product by removing the surface from the sample or removing the sample from the surface.

36. The process according to claim 25, wherein the meniscus is passed over the coupled product by evaporation of the sample.

37. The process according to claim 25, further comprising subjecting the coupled product to a stress to destroy any mismatches before detecting the macromolecule.

38. A process for detecting a macromolecule in a sample, wherein the process comprises:

(a) providing a support having a surface;

(b) anchoring a molecule with biological activity to the surface, wherein the molecule with biological activity comprises biotin, a protein, a nucleic acid, a lipid, or a polysaccharide;

(c) passing a meniscus over the molecule with biological activity to align the molecule with biological activity on the surface;

(d) contacting the sample with the surface to couple the macromolecule in the sample to the molecule with biological activity; and

(e) detecting, either directly or indirectly, the macromolecule in the coupled product from step (d).

39. The process according to claim 38, wherein the protein comprises an antibody, an antigen, a ligand, or a ligand receptor.

40. The process according to claim 38, wherein the surface comprises an organic polymer, an inorganic polymer, a metal, a metal oxide, a sulfide, or a semiconductor element.

41. The process according to claim 38, wherein the surface comprises glass, surface-oxidized silicon, gold, graphite, molybdenum sulfide, or mica.

42. The process according to claim 38, wherein the support comprises a plate, a bead, a fiber, or a particle.

43. The process according to claim 38, wherein the surface of the support comprises an exposed reactive group having an affinity for the molecule with biological activity.

44. The process according to claim 38, wherein the surface comprises a vinyl, an amine, a carboxyl, an aldehyde, or a hydroxyl group.

45. The process according to claim 43, wherein the surface of the support comprises a substantially monomo-

lecular layer of an organic compound and wherein the substantially monomolecular layer of the organic compound comprises:

(a) an attachment group having an affinity for the support; and

(b) an exposed group having no or little affinity for the support and the attachment group under attachment conditions, but having an affinity for the molecule with biological activity.

46. A process according to claim **43**, wherein the exposed reactive group is an ethylenic double bond, a vinyl group, or an amine group.

47. The process according to claim **38**, wherein the protein comprises avidin or streptavidin.

48. The process according to claim **38**, wherein the meniscus is passed over the molecule with biological activity by removing the surface from the sample or removing the sample from the surface.

49. The process according to claim **38**, wherein the meniscus is passed over the molecule with biological activity by evaporation of the sample.

50. The process according to claim **38**, further comprising subjecting the coupled product to a stress to destroy any mismatches before detecting the macromolecule.

51. A process for quantifying the amount of a macromolecule in a sample, comprising:

a) providing a support having a surface, wherein the surface comprises a molecule with biological activity attached thereto and wherein the molecule with biological activity comprises biotin, a protein, a nucleic acid, a lipid, or a polysaccharide;

(b) contacting the sample with the surface to couple the macromolecule in the sample to the molecule with biological activity;

(c) passing a meniscus over the coupled product from step (b) to align the coupled product on the surface; and

(d) detecting, either directly or indirectly, the macromolecule in the coupled complex; and

(e) quantifying the amount of the macromolecule in the sample.

52. A process for quantifying the amount of a macromolecule in a sample, comprising:

(a) providing a support having a surface, wherein the surface comprises a molecule with biological activity, wherein the molecule with biological activity has been aligned on the surface by passage of a meniscus over the molecule with biological activity and wherein the molecule with biological activity comprises biotin, a protein, a nucleic acid, a lipid, or a polysaccharide;

(b) contacting the sample with the surface to couple the macromolecule in the sample to the molecule with biological activity; and

(c) detecting, either directly or indirectly, the macromolecule in the coupled product from step (b); and

(d) quantifying the amount of the macromolecule in the sample.

53. The process according to one of claims **1, 14, 25, 38,** or **51**, wherein the molecule with biological activity is a nucleic acid.

54. The process according to one of claims **1, 14, 25, 38,** or **51**, wherein the molecule with biological activity is a protein.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,548,255 B2
DATED : April 15, 2003
INVENTOR(S) : David Bensimon, Aaron Bensimon and Francois Heslot

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 21,
Line 58, after "an antigen," insert -- a ligand, or --.

Signed and Sealed this

Twelfth Day of August, 2003

JAMES E. ROGAN
Director of the United States Patent and Trademark Office